

MECHANISM OF ACTION OF A SERUM ONCOLYTIC PROTEIN,  
RABBIT TUMOR NECROSIS FACTOR

By

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A soluble oncolytic protein, tumor necrosis factor (TNF), can be induced to appear in the serum of experimental animals which have been infected with viable Mycobacterium bovis, strain BCG, and then challenged by an intravenous administration of bacterial lipopolysaccharide (LPS) in two weeks. TNF is capable of selectively killing several transformed cells in vitro but no toxic effects on normal cells are observed. TNF also shows necrotizing activity against a variety of transplantable tumors in vivo.

Rabbit TNF has been purified by a series of salt precipitation, gel filtration, ion exchange chromatography and lectin affinity chromatography to a single homogeneous species on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). TNF activity could be recovered from non-denaturing gel systems and has been shown to be an  $\alpha$ -globulin with an isoelectric point of 5.1. The molecular weight was estimated to be 68,000 by SDS-PAGE, 55,000 by gel filtration, and 52,000 by glycerol gradient centrifugation. TNF activity was stable over the pH range of 6-10 and was relatively heat stable, not being inactivated at 70<sup>0</sup> C for 1 hour. TNF activity was pronase sensitive

but relatively trypsin resistant. Neuraminidase and phospholipase C treatment did not destroy TNF activity. Partially purified TNF was still capable of eliciting hemorrhagic necrosis in susceptible tumors. Crude TNF serum had an interferon titer of 3000 units while the partially purified sample had a titer of less than 30 units.

Rabbit TNF was also examined for its effects on transformed and normal cells in culture. Several assays for TNF activity were developed and their sensitivities and precisions compared. TNF killing of L-929 cells was delayed by 10 - 12 hours and thereafter showed concentration and time dependent increases in killing. Cells treated with actinomycin D or cycloheximide showed enhanced rates of killing as well as shorter lag periods.

Several cell types, both normal and transformed; were tested for their sensitivity to TNF. Normal cells were not killed by TNF and this discrimination was shown not to be due to differences in growth rate between normal and transformed cells. A cell cycle dependent mechanism of cell killing was also excluded.

TNF killing of L-929 cells shows less than single-hit kinetics and this is argued to suggest an enzymatic mode of action. Inhibitors of various protease classes were screened for their effect on TNF killing. None of the serine esterase inhibitors blocked TNF action; however the metal chelator o-phenanthroline did abolish TNF activity. Killing activity was restored with copper, molybdenum, and cobalt salts.

Inhibitors of receptor-mediated endocytosis such as colchicine, vinblastine, or cytochalasin B were capable of protecting target cells to killing by TNF, suggesting that the protein may have an intracellular site of action. Chloroquine, a lysomotropic agent, also protects cells against killing by TNF.



These data are argued to suggest that TNF may be a metal containing enzyme which has to gain entrance to the intracellular millieu to express its killing activity. Lysozomal enzyme functioning may be important for the ultimate expression of killing, perhaps through a protein processing step.

## INTRODUCTION

Many elements of host defense contribute to tumor cell killing and the scavenging of emerging aberrant clones of cells is becoming recognized as one of the most important functions of the immune system (Thomas, 1959, Burnet, 1967, 1970). How malignant cell killing proceeds in vivo is still largely unknown but based on several model in vitro systems appears to be multifactorial. Specifically immune T-cells (Hellstrom et al., 1971; Brunner and Cerottini, 1971), natural killer (NK) lymphocytes (Herberman et al., 1975) with or without B or T cell markers, antibody dependent cytotoxic T-cells (Pollack et al., 1972), macrophages with specific antibody (Bennett et al., 1963) activated macrophages (Cleveland et al., 1974) and polymorphonuclear leukocytes (Gale and Zigheboim, 1975) are all possible effector cells. Antibody with complement (Hellstrom et al., 1968), lymphotoxin (Granger and Kolb, 1968) produced by lymphocytes, tumor necrosis factor (Carswell et al., 1975) produced by macrophages, as well as other soluble mediators are also candidates for tumor cell killing because of their demonstrated oncolytic capabilities. Interferons, produced by T and B lymphocytes as well as macrophages during immune reactions, are known to have cytostatic effects on cells in culture, suppress immune reactions, activate macrophages, and have some anti-tumor effects in vivo (Gresser and Bourali, 1970; Gifford and Tobey, 1977; Huang et al., 1971). The study of the immune system in tumor regression is made more complex by observations that tumor cells may produce factors which subvert normal immune mechanisms (Mansfield et al., 1977; Huget et al. 1977; Friedman et al., 1976; Snyderman and Pike, 1976).

The purpose of this report is to describe one of the soluble mediators, tumor necrosis factor (TNF), and how it might contribute to tumor cell killing. Although Lewis' initial thoughts on a surveillance mechanism as formalized by Burnet are essentially cellular in nature the selective toxicity of TNF for tumor cells compared to normal cells suggests that this substance could also play an integral role in host defense against neoplasia.

### Historical

#### Events Leading to the Discovery of TNF

The anti-tumor effects of bacterial cells and their products have been known for over one hundred years and have been investigated by Bruns (1868) and by Coley (1891) who published pioneering studies describing the spontaneous regressions of certain human tumors during bacterial infections. In particular, erysipelas (reviewed by Nauts et al., 1953) and tuberculosis (reviewed by Pearl, 1929) occasionally caused a modifying, curative, or even a preventive effect on tumors. Coley, and others, subsequently developed bacteria-free filtrates from cultures of streptococci (isolated from erysipelas) and other organisms, particularly Serratia marcescens, to be used in the treatment of human malignancy. These "Coley's Toxins" were employed for over forty years with favorable results in a significant number of cases (reviewed by Nauts et al., 1953).

The study of bacterial toxins in experimental cancer seems to have received little attention until Gratia and Linz (1931) demonstrated hemorrhagic necrosis of a transplanted liposarcoma in guinea pigs using Escherichia coli culture filtrates. Soon afterwards Schwartzman and Michailovsky (1936) obtained hemorrhagic necrosis upon

parenteral injection of filtrates from meningococcal cultures into mice bearing sarcoma 180 and some of the tumors receded completely.

The parenteral administration of bacterial products to experimental animals, or human patients, may produce a severe hemorrhagic reaction within the tumor. The anti-tumor effect is rapid (about 4 hours) and confined to the core of the tumor which darkens and is eventually sloughed off. When administered in sufficient amounts, these toxins cause disseminated systemic effects leading to circulatory collapse and death. The rapid hemorrhagic reaction is followed by a more slowly progressing necrotizing reaction at the tumor site which increases in intensity over the next forty-eight hours.

Since these filtrates were quite toxic to the recipients, Shear and Andervont (1936) attempted to separate the hemorrhage producing substance from the toxic component. Shear and Turner (1944) eventually isolated a "polysaccharide," which contained some lipid and is now known to be endotoxin, from Serratia marcescens culture filtrates which could elicit hemorrhagic necrosis of both experimental and primary subcutaneous tumors (Shear 1944). Endotoxin today is known to have a large number of biological effects in the recipient including stimulation of the reticuloendothelial system, activation of macrophages, mitogenicity for B lymphocytes, enhancement of antibody synthesis, induction of interferon synthesis, as well as fever, leukopenia followed by leukocytosis and diverse vascular disturbances (Elin and Wolff, 1976; Kass and Wolff, 1973; Nowotny, 1969). An important feature of endotoxin induced tumor regression is that although the necrotic reaction is a common consequence of toxin administration, complete tumor regression is rare. Typically a ring of viable tumor tissue survives to eventually grow and kill the host.

Subsequent injections of endotoxin are usually less effective since the animal becomes refractory to the tumor inhibiting action as well as the other biological effects.

A favored mechanism for endotoxin induced tumor destruction, based on the work of Algire et al. (1952), was one of endotoxin induced systemic hypotension leading to collapse of the tumor vasculature followed by anorexia and tumor cell death. Tumor cells are not killed in tissue culture when grown in the presence of endotoxin, further suggesting that they are affected indirectly as a result of endotoxic reactions in the host. Subsequent work by several research groups suggests that additional elements, both humoral and cellular, are involved in the hemorrhagic reaction as well as the subsequent tumor regression.

Recently Berendt et al. (1978a) have proposed a model for endotoxin induced tumor regression whose central precept is the requirement for sensitized T cells. Endotoxin induced regression of established, syngeneic murine tumors depends on the generation of a state of concomitant tumor immunity (Berendt et al., 1978b). It was suggested that within this context endotoxin might more accurately be viewed as an immunotherapeutic agent. The enhanced phagocytic capacity of the reticuloendothelial system during the growth of various transplanted tumors has been established (Biozzi et al., 1958, Old et al., 1959). In addition, certain agents that are active in the reticuloendothelial system have been shown to alter the development of experimental tumors (Ribi et al., 1975; Halpern et al., 1974; Hibbs et al., 1972; Cleveland et al., 1974; Alexander and Evans, 1971; Hibbs, 1974).

Thus, when tumor bearing animals are treated with such other well known immunostimulatory agents such as Mycobacterium bovis, strain BCG

(recently reviewed by Baldwin and Pimm, 1978) or Corynebacterium parvum, significant regression rates may be obtained (Ribi et al., 1975; Halpern et al., 1974). One of the first of these studies by Old et al. (1960) used BCG infected mice, subsequently challenged with Sarcoma 180, carcinoma 755, or Ehrlich ascites cells. Mice inoculated with S-180 usually die (80-95%) in 2 to 5 weeks, the remaining regress completely. If mice were prophylactically treated with BCG significant protection was observed; up to 100% survival for those animals who were infected with BCG for 25 or more days prior to tumor challenge. The two other tumors did not show such dramatic results but the tumors did grow slower, and the mice survived for longer periods when preinfected with BCG.

Ribi and co-workers (1975) showed a synergistic effect of BCG and endotoxin in guinea pigs bearing hepatocarcinomas. The combination of live or cell wall extracts of BCG in conjunction with endotoxin was a particularly efficacious combination, with cure rates of up to 90%. This compared with regression rates of 66% for the BCG alone group.

#### The Discovery of TNF

O'Malley et al. (1962) reported the production of a tumor necrotizing factor in the serum of normal mice which had been challenged with endotoxin from Serratia marcescens. Activity was assayed by interperitoneal injection of the serum into other mice bearing sarcoma 37. The appearance of this activity in serum was rapid, short-lived, and refractory to further stimulation by repeated doses of endotoxin. Carswell et al. (1975) have also demonstrated a necrotizing factor in the serum of mice when challenged with endotoxin. However, the mice had to be primed first with BCG or other activators of the reticuloendothelial system for 1 to 3 weeks prior to endotoxin challenge

in order for the factor to be elicited. This factor, which they called tumor necrosis factor caused hemorrhagic necrosis of Meth A sarcoma when passively transferred to tumor bearing mice. TNF was shown to be active in cultures of transformed cells. The difference in these two reports, i.e. the need for a priming agent, is not clear at this time but may be due to differences in strains of mice or endotoxin preparations. Butler et al. (1978) have shown that tumor necrosis can be passively transferred by serum from normal animals injected with endotoxin. They obtained a higher titer of this serum factor when animals infected with BCG were employed. The increase in their serum necrosis factor in BCG-infected animals may be related to the findings of Suter and Kirsanow (1961) that such animals become exquisitely sensitive to the effects of endotoxin. Their serum factor was not capable of killing the tumor cells in vitro. However, they performed their in vitro assays for only 24 hours and, as we will show later, the killing effects of TNF on cells in culture are delayed and maximal killing occurs between 24 and 48 hours. An important contribution of this study was the finding that a non-toxic polysaccharide-rich product obtained by acid hydrolysis of endotoxin was capable of eliciting the serum factor which causes tumor regression.

#### Definition of TNF

TNF is defined as a substance found in serum of animals sensitized to BCG (or certain other immunopotentiators) and challenged later with endotoxin and which causes the necrosis of some tumors when passively transferred to tumor bearing animals. TNF has other unique characteristics such as lack of species specificity and the ability to discriminate between normal and certain tumor cells in vitro. The in vitro cell killing effects of TNF are assumed to be due to the same

factor as that which causes hemorrhagic necrosis in vivo. Whether this assumption is justified will have to await final purification. It is reasonable that TNF activity measured in vitro is one component of a complex host-tumor interaction leading to hemorrhagic necrosis.

#### Priming and Elicitation of TNF in vivo

TNF was first reported to be found in the sera of mice sensitized to BCG and challenged two to three weeks later with endotoxin (Carswell et al., 1975). Rats and rabbits also produce TNF under similar conditions. The activity was not found in the sera of mice or rabbits given either BCG alone or endotoxin alone. The optimal time for collection of serum for maximal TNF is two hours following endotoxin injection; it is not found six hours later. BCG is not the only successful priming agent; heat killed Corynebacterium parvum or Corynebacterium granulosum or yeast cell walls (zymozan), which also produce hyperplasia in the reticuloendothelial system, are as effective as BCG (Green et al., 1977).

Agents other than endotoxin have been shown to elicit TNF in BCG primed mice (Carswell et al., 1975). These include a mixed bacterial vaccine consisting of heat-killed Streptococcus pyogenes and Serratia marcescens. It is probable that the endotoxin from Serratia was responsible for the elicitation of TNF. However, Brucella abortus, which also contains endotoxin, did not elicit TNF in BCG primed mice. This may be due to the observation that Brucella abortus endotoxin is not very toxic. Old tuberculin was also ineffective in inducing TNF.

The strain of mouse is important. Old (1976) has reported that BP 8 tumors grew equally well in two strains of C3H mice known to differ in their response to endotoxin as measured by B cell mitogenesis and



lethality. Only the endotoxin sensitive strain showed tumor necrosis when injected with endotoxin. However, both strains of mice showed tumor necrosis when TNF was injected. TNF can be produced in BCG infected mice by endotoxin in endotoxin sensitive mice (C3H/HeN) but not in endotoxin insensitive mice (C3H/HeJ) (Mannel et al., 1979b). Furthermore, these authors have shown that the ability to release TNF could be transferred into lethally x-irradiated endotoxin nonresponders by reconstitution with endotoxin responder bone marrow cells.

Some studies have shown that a cytotoxic factor can be produced by cultured macrophages under certain conditions that do not require prior exposure to BCG. However, these factors have not been tested for their ability to cause tumor necrosis in vitro and may be different from TNF. These will be discussed in the next section.

#### Cells Responsible for TNF Production

##### Implication of the Macrophage

In their original paper (Carswell et al., 1975), the authors speculate that the cellular origin of TNF is probably the macrophage. They point out that massive hyperplasia of macrophages occur in the spleen of BCG-infected mice and that pyknosis and disruption of the population occurs two hours after the administration of endotoxin. This is further supported by numerous observations that mouse peritoneal macrophages can be activated by a variety of diverse stimuli to become broadly cytotoxic for syngeneic, allogeneic, or xenogeneic tumor cells but not for normal cells (Hibbs, 1974; Piessens et al., 1975). These mechanisms include chronic infection with BCG or exposure to endotoxin (Cleveland et al., 1974), double stranded RNA (Alexander and Evans, 1971) as well as co-cultivation with sensitized lymphocytes and

antigen (Evans and Alexander, 1971) or by the addition of lymphokines to peritoneal cells (Piessins et al., 1975).

Old (1976) points out that TNF could not be induced in athymic nude mice (nu/nu) when primed with Corynebacterium parvum and subsequently injected with endotoxin suggesting participation of T lymphocytes. However, more recently, Mannel et al. (1979b) have found that serum from nude mice infected with BCG and treated with LPS contained as much cytotoxicity as did their heterozygous littermates. Perhaps, there is a difference in the ability of the two priming agents (BCG and C. parvum) to prime for TNF production in athymic mice.

Many cytotoxic factors in the supernatant fluids of macrophage cultures from human, guinea pigs, rats and mice have been described over the past 12 years (Table I). Some of these factors are spontaneously produced in culture but others are induced by such substances as endotoxin and purified protein derivative (PPD) from old tuberculin either in normal or sensitized animals. However, in some of the inducible systems, a background level of activity is found in supernatant fluids without inducer. Weinberg et al. (1978) have reported that many reagents are contaminated with endotoxin including calf serum. Thus, it is possible that the "nonstimulated" cultures were actually stimulated with this contaminant.

When tested on normal cells, the macrophage supernatants are sometimes cytotoxic indicating that they are dissimilar to tumor necrosis factor and similar to lymphotoxins in this regard. For example, Kramer and Granger (1972) employing macrophages from sarcoma sensitized mice found a toxic factor released into the medium when exposed to alleneogenic target L cells. Medium from cultures of normal cells plus target cell or normal cells alone had a low level of activity. The

TABLE I  
Macrophage Toxins

Investigator:	Sintex and Pincus (1970), Pincus (1967)	Heise and Weiser (1969)
Species:	Normal and tuberculin sensitive guinea pigs	Normal and tuberculin sensitive guinea pigs
Cell Source:	Adherent peritoneal exudate cell, thioglycollate induced	Lung lavage
Inducer:	PPD, PHA, others	Spontaneous but increased with PPD
Time of Production:	1/2 to 4 hours	Collected at 24 hours
Assay:	L, HeLa; cell killing (dye exclusion)	L cells, enumeration after 24 hours
Species Specific:	No	*
Toxicity for Normal Cells:	Yes	*
Stability:	100 C/60 min.	80 C/30 min.
Molecular Weight:	10,000	Two peaks by gel filtration
Characteristics:	Phospholipid, usually associated with protein	Similarity to lymphocyte cytotoxicity induced by PPD

TABLE I--Continued

Investigator:	Kramer and Granger (1972)	Currie and Basham (1975), Currie (1978)
Species:	Mouse sensitized to sarcoma	Normal rats and mice
Cell Source:	Adherent peritoneal cells	Adherent peritoneal cells
Inducer:	L cells	Endotoxin, also spontaneous
Time of Production:	Collected at 48 hours	18 hours, then increased
Assay:	<sup>14</sup> C-amino acid incorporation, 72 hours	Rat sarcoma, <sup>125</sup> I <sub>I</sub> others; release of release of
Species Specific:	No	No
Toxicity for Normal Cells:	Yes	No
Stability:	100 C/15 min.	"Labile"
Molecular Weight:	47K, 150K	*
Characteristics:	Protein, lymphotoxin like	Arginase activity

TABLE I--Continued

Investigator:	Reed and Lucas (1975)	Jones et al.
Species:	Normal rats and humans	Mouse, normal or <u>C. parvum</u> sensitized
Cell Source:	Adherent peritoneal, spleen and blood mononuclear cells	Adherent peritoneal cells induced with <u>C. parvum</u>
Inducer:	Spontaneous	Spontaneous, but increase with <u>C. parvum</u>
Time of Production:	4-8 hours	*
Assay:	L, others; 3H-thymidine incorporation	51 Cr release from normal cells at 4 hours
Species Specific:	No	*
Toxicity for Normal Cells:	No	Yes
Stability:	*	*
Molecular Weight:	45K	*
Characteristics:	Protein, not lymphotoxin like	*

TABLE I--Continued

Investigator:	Matthews (1978)	Aksamit and Kim (1979)
Species:	Normal rabbits	Mouse
Cell Source:	Adherent blood mononuclear cells	Transformed macrophage cell lines
Inducer:	Spontaneous	Spontaneous
Time of Production:	3-7 hours	Maximum at 7 days
Assay:	L cells, killing after 72 hours	<sup>3</sup> H-thymidine release from fibrosarcoma cells
Species Specific:	No	*
Toxicity for Normal Cells:	No	*
Stability:	*	*
Molecular Weight:	30K-50K	40K-50K, 200K
Characteristics:	Protein, similarities to TNF	*

TABLE I--Continued

Investigator:	Mannel et al. (1979a)	Mannel et al. (1979a, b)
Species:	BCG Infected mice	Normal mice
Cell Source:	Adherent peritoneal cells	Adherent peritoneal cells treated with lymphokines
Inducer:	Endotoxin	Endotoxin
Time of Production:	1-2 hours	2 hours
Assay:	<sup>3</sup> H-thymidine release from L cells	<sup>3</sup> H-thymidine release from L cells
Species Specific:	*	*
Toxicity for Normal Cells:	No	No
Stability:	56 C/30 min.	56 C/30 min.
Molecular Weight:	55K-60K <sup>a</sup>	55K-60K <sup>a</sup>
Characteristics:	--	--

TABLE I--Continued

Investigator:	Mannel et al. (1979b)
Species:	Mouse
Cell Source:	Macrophage cell line
Inducer:	Endotoxin
Time of Production:	2 hours
Assay:	<sup>3</sup> H-thymidine release from L cells
Species Specific:	*
Toxicity for Normal Cells:	No
Stability:	56 C/30 min.
Molecular Weight:	55K-60K <sup>a</sup>
Characteristics:	--

\*Not reported.

<sup>a</sup>Antibody to partially purified TNF neutralized these factors.



factor was also toxic to normal mouse embryo fibroblasts. Two fractions of toxic activity were eluted from Sephadex which had approximate molecular weights of 150,000 and 47,000. Antiserum prepared against partially purified PHA-induced mouse lymphotoxin was capable of neutralizing both the lymphotoxin and the macrophage cytotoxin indicating that the factors may be similar. Heat stability (100°C, 15 minutes) was another feature in common with this lymphokine. Table I lists the inducers and gives some properties of many of these factors. In those studies where molecular weights have been estimated by gel filtration on Sephadex the molecular weights have usually been in the range of 40,000 to 50,000. Some have an additional cytotoxic component whose elution characterizes a molecule of approximately 150,000 daltons. Thus there are similarities with the factor described by Kramer and Granger (1972) relative to molecular weight. One factor of considerably lower molecular weight has been reported. Pincus (1967) has described a factor produced by macrophage cultures from normal guinea pigs when challenged with PPD, bovine serum albumin or other antigens, or the mitogen PHA. Occasionally low levels of activity could be found in non-stimulated cultures. In a later paper (Sintex and Pincus, 1970), the authors used thioglycollate stimulated peritoneal cells from tuberculin-positive guinea pigs and induced the factor with PPD. Cytotoxic activity from these supernatants chromatographed on Sephadex with an estimated molecular weight of 30,000- 40,000. It was found to be heat stable and could be boiled for 1 hour without losing activity. Upon further purification, the activity was dissociated from protein and the activity now chromatographed at 1000d. It was inactivated by acid phosphatase and phospholipase D and was resistant to pronase and is probably a phospholipid.

Cytotoxic activity has been found in the culture fluid from 4 of 5 macrophage cell lines (Aksamit and Kim, 1979). The factor was active on a fibrosarcoma cell line; activity on normal cells was not reported. Using Sephadex G-200 filtration, the activity was in the 40,000 to 50,000 d range with a second peak in the void volume.

Evidence that products secreted by stimulated lymphocytes activate macrophages to become tumoricidal has been published by several laboratories (e.g., see Piessens et al., 1975). Leonard et al., (1978) have purified a lymphokine called macrophage activation factor (MAF) from spleen cells from BCG-infected mice which were incubated with PPD. This factor makes macrophages nonspecifically cytotoxic for tumor cells.

Since the discovery and characterization of TNF there has been increasing concern whether the macrophage cytotoxins are related to TNF. Matthews (1978) has shown that apparently non-activated adherent mononuclear cells from normal rabbit blood were cytotoxic to those cell lines in vitro which were also susceptible to TNF and were not cytotoxic for TNF resistant cells. The bulk of the activity was expressed by monocyte-enriched fractions. Detectable amounts of TNF-like activity were found after 3 hours in culture and maximal amounts by 7 hours. Other similarities of the monocyte cytotoxic factor and TNF were studied: both were precipitated with 50% ammonium sulfate, eluted on gel filtration in the range 30,000-50,000 d, and migrated in the same position by polyacrylamide gel electrophoresis.

Männel et al. (1979 a,b) have studied four cytotoxins from macrophages. These were cytotoxic factors found in the supernatants from BCG activated macrophages incubated with endotoxin, a macrophage cell line incubated with endotoxin, a cytotoxin from thioglycollate induced

peritoneal cells propagated in the presence of supernatant fluids from mouse L-929 cells and then stimulation with endotoxin, and cultivation of peritoneal cells in lymphokine medium (from Con A stimulated spleen cells) followed again by induction with endotoxin. The molecular weight of all cytotoxins as detected by gel filtration was approximately 55,000 to 60,000 and the activity of all four eluted from DEAE-Sephacel under similar conditions. Thermostability was similar. Antibody prepared in rabbits against partially purified TNF was able to neutralize the cytotoxicity of the 4 cytotoxins as well as TNF. These data suggest but do not prove that cytotoxins produced by macrophages under different conditions may be similar and related to TNF.

Are macrophage cytotoxins equated to TNF?

Considerable reservations have to be made in assuming that factors from cell cultures are equated to TNF. For example, Currie and Basham (1975) have shown that a soluble supernatant factor can be found in the supernatant of rat and mouse macrophages which lyses malignant but not normal cells. Currie (1978) subsequently has shown that this factor has arginase activity and that the cytotoxic effects could be inhibited by adding excess arginine. Currie and Basham (1978) have recently shown that malignant cells require a higher concentration of L-arginine in the medium than the normal counterparts and that complete arginine deprivation caused a more rapid cytolysis of the malignant cells. They also suggested that preliminary studies indicated that macrophages freshly isolated from tumors contain high levels of arginase. We know that TNF killing is not influenced by adding excess arginine or any other amino acid (vide infra). The possibility of nutrient depletion has not been shown for any other of the other factors except for the

lymphotoxin-like factor from macrophages reported by Kramer and Granger (1972).

## MATERIALS and METHODS

### Methods

#### Preparation of TNF Containing Serum.

New Zealand white female rabbits (NZW) weighing from 2-3 kg. were bled by cardiac puncture as a source of normal rabbit serum (NRS) for control experiments. Rabbit tumor necrosis factor (TNF) was produced by the procedure by Carswell et al. (1975). Viable Mycobacterium bovis, BCG (Tice stain,  $3 \times 10^8$  organisms) were injected into the marginal ear vein. Fourteen days later, 100  $\mu$ g of endotoxin from Salmonella typhimurium, virulent strain 7, was injected into the ear vein. The endotoxin was a gift from Dr. Joseph Shands, University of Florida. Rabbits were sacrificed by cardiac puncture 1.5 hours later. Blood samples were also taken prior to injection of endotoxin, and endotoxin was also administered to non-BCG infected rabbits. The endotoxin alone did not demonstrate any TNF activity.

#### Cells.

L-929 is a mouse transformed cell line originally derived from the C3H strain of mouse. Cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% bovine serum and 250 units of penicillin/ml and 125  $\mu$ g of streptomycin/ml. Primary fibroblast cultures from mouse, chicken, and hamster embryos and rabbit kidney cultures were prepared by minicing near term embryos or mature kidneys, trypsinization (0.1%) with 0.04% ethylenediamine tetraacetic acid (EDTA) for 60 minutes, followed by washing with Gey's balanced salt solution (BSS) and dispensing the cells in glass bottles containing fortified MEM with 10% fetal bovine serum (FBS). Fortified MEM contained 2x concentrations of essential amino acids, non-essential amino acids,

vitamins, and glutamine. Sodium pyruvate was also added (Dion et al., 1977). Flow 7000 are human embryonic foreskin fibroblasts obtained from Flow laboratories. B16C3 is a mouse melanoma HeLa is a human cervical carcinoma. AV3 is a human amnion cell.

#### Assays for TNF.

TNF was assayed by several methods in order to better evaluate the sensitivity and precisions of the various techniques. These assays are similar to others which have been developed to study the actions of other cytotoxins, most notably lymphotoxin (Sawada and Osawa, 1977; Henney, 1973; and Kramer and Granger, 1972). In the morphological microassay, cultures of cells are established in 96 well flat-bottomed trays (3040, Falcon Plastics, Oxnard, CA.) at 50,000 cells/0.2 ml in a humidified atmosphere at 37° with 5% CO<sub>2</sub>, unless noted otherwise. Cells are typically added at 2 x concentration in a volume of 0.1 ml to dilutions of TNF are control serum in media in a volume of 0.1 ml. Forty-eight hours later, media is decanted, and residual cells are stained with crystal violet and cells in representative microorganic fields are counted. A modification of this basic assay was to pre-establish cells in 0.1 ml for 15 hours, then TNF dilutions and actinomycin D at 1 µg/ml final concentration was added to a final volume of 0.2 ml. Plates were stained after 18 hours. TNF killing was also determined by establishing cells in 25 cm<sup>2</sup> flasks for 18 hours followed by addition of TNF or control serum. Specific plating conditions are indicated in the figure legends. Morphologically intact cells were determined at various times by cell counting

For the cytotoxic release assay L-929 cells were grown for three days in the presence of 1.0 µCi/ml [<sup>3</sup>H] thymidine (6Ci/mmmole, Schwarz/Mann). Cells were washed three times with warm MEM prior to

replating in 25 cm<sup>2</sup> flasks, as above. Cytolysis was measured at various times by sampling aliquots of centrifuged supernatant fluids for scintillation counting. The specific lysis was computed by the formula:

$$\text{Specific lysis} = \frac{\text{Experimental} - \text{Background (CPM)}}{\text{Control} - \text{Background (CPM)}}$$

The 100% releasable CPM was determined by incubating target cells in 0.5% SDS for 30 minutes. Spontaneous release was 8-14% of maximal release. The mean and standard deviation of triplicate samples were determined.

In the "end label" method cells were established in microtiter trays as for the morphological assay. At 44 hrs after TNF addition the cultures were pulsed with 0.5  $\mu\text{Ci}$  [<sup>3</sup>H] thymidine (56 Ci/ $\mu\text{mole}$ , Schwarz/Mann) for 4 hours. Cells were collected onto glass fiber filter strips with an automated sample harvester and radioactivity counted. The means and standard deviations of triplicate samples were determined.

#### Cell Counting.

Cell counting was carried out under an inverted microscope at 200x. Numbers of morphologically intact cells were determined by use of a micrometer mounted in the ocular (5 x 5 mm<sup>2</sup>). Cells were counted at several points on the field until 300 cells or nine grid squares were counted. Cell killing is expressed as a survival ratio, S/So, where So = number of cells in control culture and S = number of cells in experimental cultures.

## Purification and Physical-chemical Characterization of TNF

### Ammonium Sulfate Fractionation

Saturated ammonium sulfate (SAS) was added to crude TNF serum and the pH adjusted to 7.0 with  $\text{NH}_4\text{OH}$ . Samples were left to precipitate overnight at 4°C and then centrifuged at 10,000 X G for 30 minutes in the cold to collect pellets and supernatant fluids. Pellets were reconstituted with 50 mM phosphate buffer, pH 7.5.

### Ultragel AcA 34 Gel Filtration

Protein from the ammonium sulfate precipitation procedure was loaded onto an AcA 34 (LKB, Stockholm) column, 5 X 60 cm. The column was eluted with 50 mM phosphate buffer, 120 mM NaCl, pH 7.0. Flow rate was 10 ml/hr and 12 ml fractions were collected. This procedure and all successive purification steps were carried out at 4°.

### DEAE - Sephadex Chromatography

Fractions with TNF activity from the AcA 34 column were pooled, concentrated by pressure ultrafiltration and loaded onto a DEAE-Sephadex column (Pharmacia, Piscataway, N.J.) 2.5 x 25 cm, previously equilibrated with starting buffer; 50 mM phosphate, 120 mM NaCl, pH 7.0. The column was washed with starting buffer until the major portion of unbound proteins washed through, approximately four column volumes. A linear salt gradient of five column volumes, from 120 to 400 mM NaCl, was used to elute TNF activity. Six milliliter fractions were collected at a flow rate of 10 ml/hr.

### CM Sephadex Chromatography

A cation exchange chromatographic step was occasionally performed subsequent to the DEAE column. This step was removed from the final purification scheme since it did not result in enhancement of overall purification. CM sephadex (10 ml packed) was



equilibrated in starting buffer of 20 mM acetate, pH 5.0. TNF dialysed into starting buffer did not bind to this column under these conditions and was therefore recovered in the wash fractions. The pH was adjusted to neutrality by collecting fractions into tubes containing small volumes of a saturated Tris-base buffer.

#### Con A Sepharose Chromatography

Fractions with TNF activity from the anion exchanger DEAE-Sephadex were concentrated and dialysed against PBS. This material was loaded onto a Con A Sepharose column with a 10 ml bed volume. Elution was with PBS since TNF was not bound by Con A. The column was regenerated by washing with  $\alpha$ -D-methylmannoside, 20 mg/ml in PBS, followed by starting buffer.

#### Sephacryl-200 Chromatography

The pooled concentrated TNF containing fraction from the Con A Sepharose step was loaded in a volume of 2.0 ml onto a Sephacryl-200 column, 1.5 x 180 cm. Elution was with 50 mM Tris, 0.15 M NaCl, 10 mM EDTA buffer, with 10% glycerol at pH 7.5. Fractions of 1.5 ml were obtained at a flow rate of 8 ml/hr. The column was separately calibrated with blue dextran, human IgG (160,000d), bovine serum albumin (68,000d), and ovalbumin (45,000d) as molecular weight markers. Fractions with TNF activity were pooled and concentrated as the final purified material.

#### Polyacrylamide Gel Electrophoresis

Molecular size and homogeneity of protein preparations was determined by electrophoresis on 10% polyacrylamide gels with sodium dodecyl sulfate (SDS-PAGE) according to the method of Laemmli (1970). Proteins with known molecular weights served as internal markers. Partially purified TNF and marker proteins

were also electrophoresed on 15% polyacrylamide gels under non-denaturing conditions. Gels were frozen and then cut into 8mm slices. Each slice was macerated in a dounce homogenizer and proteins eluted by incubation of the gel fragments in 1.0 ml of PBS with antibiotics for two days at 37° on a shaker table. Samples were clarified by centrifugation and supernatant fluids were assayed for TNF activity. This procedure was also employed for recovery of TNF activity from the isoelectric focusing gels. Visualization of proteins was done by staining with Coomassie Blue R-250, or for those cases where 125-iodinated proteins were used, by autoradiography on Dupont Cronex film.

#### Iodination of Purified TNF

Purified TNF, 5 µg in a volume of 20 µl of 0.1 M borate buffer, pH 8.5, was reacted with 10 µl of dried Bolton-Hunter reagent (4000 Ci/mmol; New England Nuclear) for 1 hour as described by (Bolton and Hunter, 1973) and product literature. Unreacted ester was then reacted with 0.5 ml of 0.1 M Tris buffer, pH 8.5 for minutes at 0° C. Cytochrome c, 100 µg was added as cold carrier proteins and samples were precipitated twice with cold acetone to remove non-macromolecular label and finally resuspended in 50 µl of PBS. A portion of the carrier protein was labeled, presumably by unreacted Bolton-Hunter reagent. Accurate values for the specific activity of labeling could therefore not be determined. Specific activity was estimated to be 5-10 µCi/µg TNF. Marker proteins, 10 µg each, were mixed and iodinated as a single batch by the same protocol.

#### Isoelectric focusing

Iodinated TNF was subjected to isoelectric focusing by a modification of the method of O'Farrel (1975) using N, n'-diallyltartardiamide (DATD) rather than Bis-acrylamide as the crosslinking

agent (Horst and Roberts, 1979; Horst et al., 1979) and deleting the solubilizing agent, NP-40. Briefly, 4% DATD-acrylamide gels with 3% pH 3.5-10 and 2% pH 5-7 ampholites were prepared by polymerization with riboflavin-TEMED and persulfate. Gels were run in 0.06 N H<sub>2</sub>SO<sub>4</sub> in the lower chamber and degassed 0.04M NaOH in the upper chamber by the following protocol: 75v for 15 min., 150v for 30 min., 300v overnight, and 750v for 1-2 hours. After focusing, gels were frozen, cut into 4 mm slices, and assayed for pH and TNF activity.

#### Glycerol gradient ultracentrifugation

Samples of purified TNF containing 125 ng of protein, or marker proteins at 1 mg/ml, were loaded in a volume of 25  $\mu$ l onto 5.0 ml glycerol gradients (20-40%) in Tris-NaCl-EDTA (Sephacryl-200 buffer). Centrifugation was performed in an SW 50.1 rotor (Beckman, Palo Alto, CA.) at 45,000 RPM for 56 hours. Six drop fractions (approximately 0.14 mls) were collected from the bottom of the gradients and assayed for TNF activity. Bovine serum albumin, ovalbumin, and chymotrypsinogen were used as molecular weight markers and were run in parallel buckets. Protein determinations were by the method of Bradford (1976).

#### Enzyme Digestions

Purified TNF was tested for sensitivity to hydrolysis by trypsin, neuraminidase, pronase, and phospholipase C. Purified TNF, 25  $\mu$ g, was incubated with 25  $\mu$ g of trypsin-TPCK (269 U/mg, Worthington, Freehold, N.J.) in 0.5 M Tris-HCl buffer, pH 8.1 with 10 mM CaCl<sub>2</sub> in a final volume of 50  $\mu$ l at 37° for 180 minutes. Extensive trypsin digestion was done with 250  $\mu$ g of trypsin incubated for 32 hours. Purified TNF, 25  $\mu$ g, was also incubated with 8  $\mu$ g neuraminidase (47 U/mg, Cl. perfringens, type IX, Sigma, St. Louis, MO)

in 50 mM acetate buffer pH 5.0, in a final vol of 50  $\mu$ l at 37° for 180 minutes. Pronase digestions (Streptomyces griseus protease, B grade, nuclease free, 97,100 P.U.K./g, Calbiochem-Behring, La Jolla, CA) were performed with 25  $\mu$ g TNF and 10  $\mu$ g enzyme in PBS, also in a reaction volume of 50  $\mu$ l for 180 minutes at 37°. Phospholipase C digestion (B. cereus, B grade, 145 U/mg Calbiochem-Behring, La Jolla, CA) were performed with 25  $\mu$ g TNF and 5  $\mu$ g of enzyme in PBS in a reaction volume of 50  $\mu$ l for 180 minutes at 37°. Control reaction mixtures for all digests were performed in the absence of added enzyme.

#### pH Stability of TNF

TNF containing serum, 1.0 ml, was mixed with 10 ml aliquots of various 50 mM salt buffers with pH ranging from 2.0 to 11.0. Samples were kept at 8° for 48 hours then diluted 1:10 with PBS prior to assay. The buffers employed were: citrate-phosphate (pH 2-6), phosphate (pH 7.0), and glycine-NaOH (pH 8-11).

#### Interferon Assays

Interferon was assayed on primary or secondary rabbit kidney cell cultures by a VSV plaque reduction method (Blalock and Gifford, 1976) except that target monolayers were established in 24 well plastic trays (Linbro, Hamden, Conn). Cells were prepared as described previously (Ruff and Gifford, 1980a).

#### Necrosis of Meth A Tumors

Meth A tumor cells were kindly provided by Dr. Saul Green, Memorial Sloan-Kettering Cancer Center, N.Y. Tumors were carried as an ascites in the peritoneum of Balb/c mice by weekly passage of 2-4 x 10<sup>6</sup> cells into naive recipients. For necrosis assays, 10<sup>6</sup> tumor cells, washed and suspended in MEM, in a volume of 0.05 ml were injected intradermally into mice to establish solid

tumors. Six to seven days later mice were injected with either normal rabbit serum, serum TNF, or partially purified rabbit TNF. Necrosis reactions were scored 24 hours later at 1<sup>+</sup> (slight necrosis confined to the center of the tumor), 2<sup>+</sup> (appreciable necrosis, encompassing at least 50% of the tumor), 3<sup>+</sup> (severe necrosis, involving the entire tumor mass). Animals were injected by tail vein in a volume of 0.3) mls. Serum TNF produced in rabbits had a titer in our 18 hour L-929 cell killing assay of 30,000 units. Partially purified TNF was DEAE purified material, lacking any interferon activity, adjusted to a titer of 30,000 units. Partially purified TNF had an interferon titer less than 30 units, the crude TNF serum had a titer of 3000 units/ml.

## RESULTS

### Assays for TNF

#### Tumor Necrosis Assay

The original assay for TNF used by Carswell et al. (1975) was a visual observation of necrosis in a subcutaneous transplant of a BALB/c sarcoma, Meth A. Grades of response (- to +++) are recorded. In the maximum response (+++) the major part of the tumor mass is destroyed leaving only a peripheral rim of apparently viable tumor tissue. The response is best and most consistently produced on well established transplants (7 days) and less effective on 6 day transplants. For some reason there is virtually no effect on 5 day transplants. This response is similar to that obtained when endotoxin is given to tumor bearing animals. Several other assays have been described.

#### In Vitro Assays

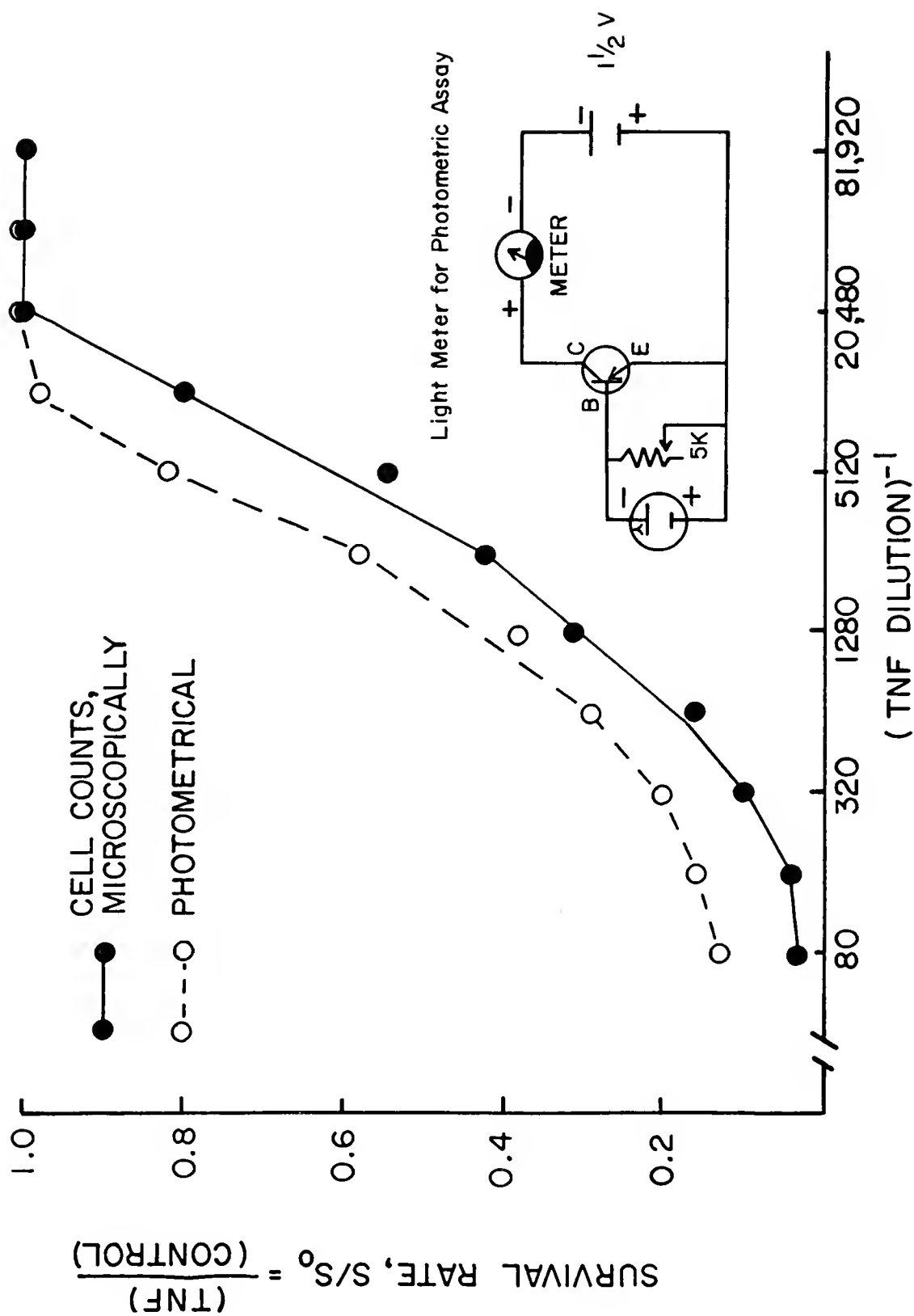
Carswell et al. (1975) used cultures of Meth A, L-929, and normal mouse embryo cells and counted the number of treated cells remaining as compared to control cultures after a 48 hour exposure to dilutions of serum containing TNF. These authors claimed that in a broad range of tests there were no discrepancies between the TNF activity against Meth A in vivo and their toxicity to L-929 cells in vitro. We have employed this procedure with L-929 cells (Ostrove and Gifford, 1979). More recently, faced with a large number of assays in order to monitor our purification procedures, we have employed 96 well microtiter plates (Falcon 3040) (Ruff and Gifford, 1980b). Briefly, 2-fold serial dilutions of TNF samples are made directly in plates which have been seeded on the previous day with 60,000 L-929 cells in each well. Alternatively, dilutions of TNF are made and then the cells added. The plates are incubated for 48 hours and the residual cells stained with crystal

violet. Visual inspection can reveal that dilution of the sample which destroys approximately 50% of the cells. This (dilution to extinction titer) is usually sufficient when relative titers are being determined. For better quantification, we count the number of cells remaining in several low-powered microscopic fields using a grid in the eyepiece. This procedure is similar to that employed by Matthews and Watkins (1978). We have recently developed an improved method of determining cell numbers based on a photometric measurement. Trays of L-929 cells are prepared as usual to establish dense monolayers. At the end of the culture period trays are stained for a uniform period of time, usually 10-15 minutes, washed with water, and allowed to dry. A photometric measuring device was constructed by cementing a selenium photocell (276-115, Radio Shack, Ft. Worth, TX) to a short piece of copper tubing (6 x 30 mm) whose outside diameter approximated the inside diameter of the micro- titer plate. A simple series circuit can be constructed using a pH meter, in the millivolt mode, to read the reference voltage drop across the photocell as a function of light intensity. Alternatively the circuit in Fig. 1 can be constructed utilizing any convenient DC milliammeter as recording device (276-103, PNP germanium transistor; 271-154, 5K potentiometer, Radio Shack, Ft. Worth, TX). Our pH meter (Corning, Model 125) has a 1 mv sensitivity. Under these conditions the resolution of the detector is 1000-1500 cells (i.e. 1mv = 1500 cells). The photocell has a linear response at least over the range 5000- 60,000 cells. The method is somewhat less sensitive than individual cell counting but enjoys advantages of speed and lack of bias in cell counting (Fig. 1).

We have shown (Ostrove and Gifford, 1979) that actinomycin D greatly enhances the cell killing of L cells by TNF. The dose response

Figure 1. Dose response curves showing the survival ratio of L-929 cells as a function of the dilution of TNF serum. Survival ratios were determined by counting cells in representative microscopic fields or by the use of a photometer.





curve for cell killing when actinomycin D and TNF are added simultaneously is shown in Figure 2 (Gifford et al., 1979). Actinomycin D shifts the dose response line two to three logs greater in sensitivity. Eifel et al. (1975) have also shown that actinomycin D enhances the cytotoxicity of lymphotoxin and have developed a similar sensitive assay for that factor. The photometric assay using cells with TNF and actinomycin D added simultaneously to microtiter plates is now used routinely in our laboratory. The procedure shortens the assay time from 48 to 18 hours and increases the sensitivity so that lower doses can be measured. We have developed two other assays for TNF activity which gives us some clues to how TNF activity may be expressed (Ruff and Gifford, 1980a). One method determines the extent of inhibition of DNA synthesis after 48 hours exposure to TNF. The "end label" technique consists of treating cultures of L-929 cells with TNF for 48 hours in microtiter plates. At the end of the incubation period, the medium is decanted and replaced with medium containing  $^3\text{H}$ -thymidine for 30 minutes and the cells then harvested with a multiple automated sample harvester. Another type of assay for TNF activity is used in which the cells are prelabelled with  $^3\text{H}$ -thymidine or carrier-free  $^{32}\text{p}$  for 48 to 72 hours prior to distribution to microtiter plates. Männel et al. (1979a) have also used a  $^3\text{H}$ -thymidine release assay. Dilutions of TNF are then added and the cultures incubated for 48 hours at  $37^\circ\text{C}$ . The released label in this "cytotoxicity release" assay is then measured and compared to control cultures. The last two assays are depicted in Figure 3 which is a composite comparison of the four methods. The "cytotoxic release" is the most sensitive followed by the "end-label" assay. The "cell-killing" assay is the least sensitive of the three assays, except when killing is measured in the presence of Actinomycin D.

Figure 2. The effect of actinomycin D, 1  $\mu\text{g/ml}$ , on the dose kinetics of TNF cell killing. Assays performed in the absence of the inhibitor were for 48 hours, while those with the inhibitor were read at 18 hours.

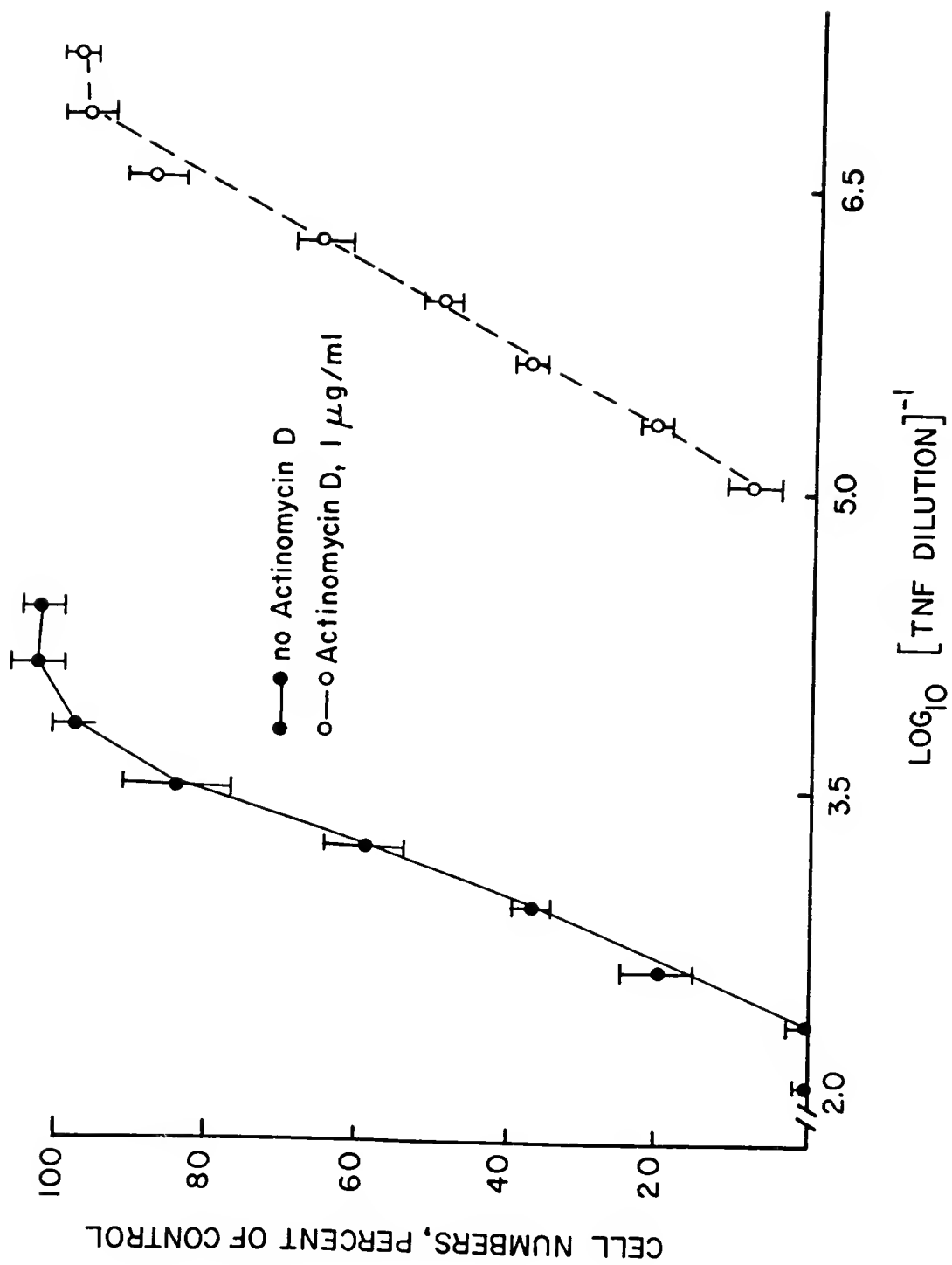
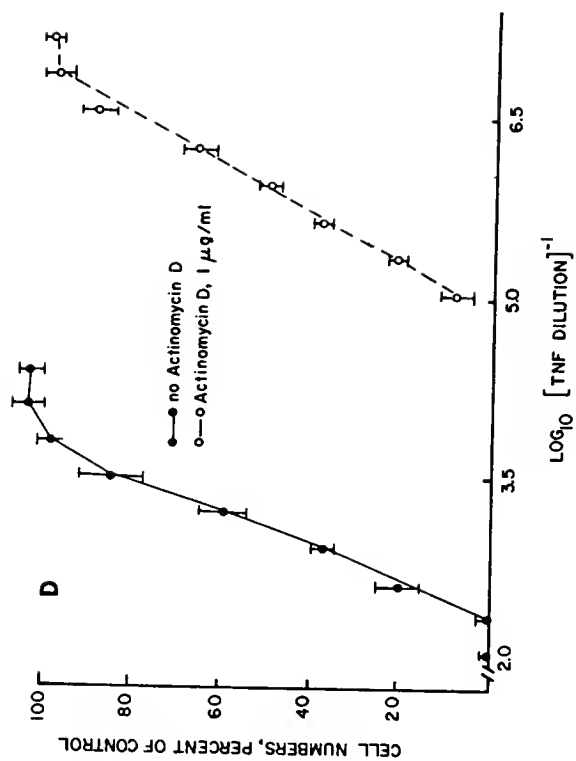
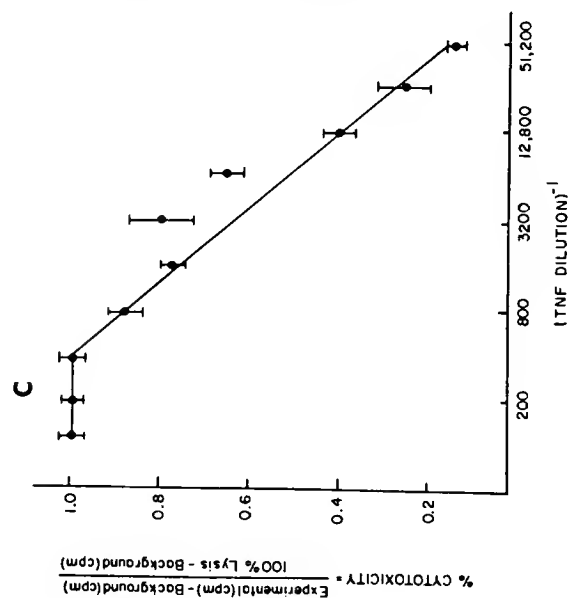
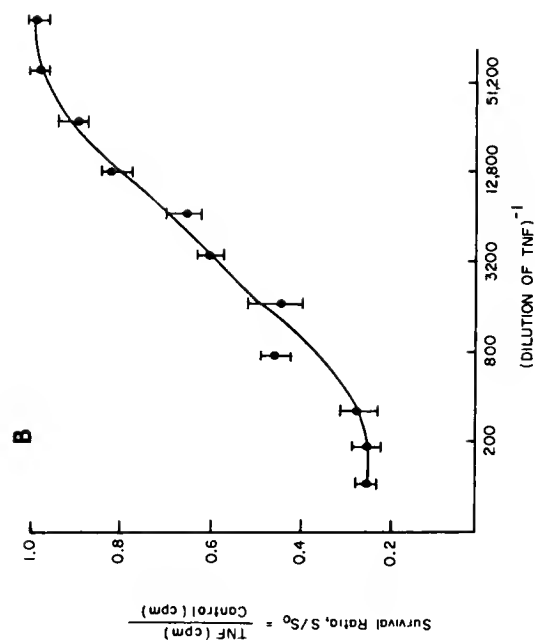
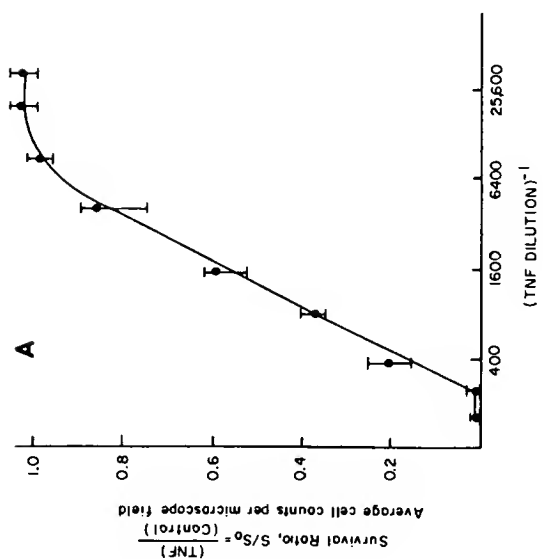


Figure 3. Dose response curves for TNF killing by several assay methods.  
Panel: A - micromorphological cell counting  
B - end-label isotope incorporation  
C - cytotoxic release from pre-labeled targets  
D - comparison of dose kinetics by the micromorphological  
method in the presence of actinomycin D.  
Cultures without the inhibitor were read at the end of 48 hours  
while inhibitor treated cultures were for 18 hours.



### Cells Susceptible to TNF Action

Using these assays, numerous transplanted tumors and transformed cells have been shown to have a high degree of sensitivity to TNF. These include: Sarcomas S-180 (CD1-Swiss) and BP8 (C3H); leukemias EL4 (C57BL/6) and ASL1 (A strain), and mastocytoma P815 (DBA/2). TNF also showed activity against several transformed lines in culture. L-929 cells were most sensitive, Meth A cells less sensitive, and normal mouse embryo fibroblasts (MEF) were insensitive. Helson et al. (1975) extended these observations and showed that human melanoma cells whose growth had been inhibited by mouse TNF when replated in the absence of TNF were still inhibited and did not resume their normal growth rate.

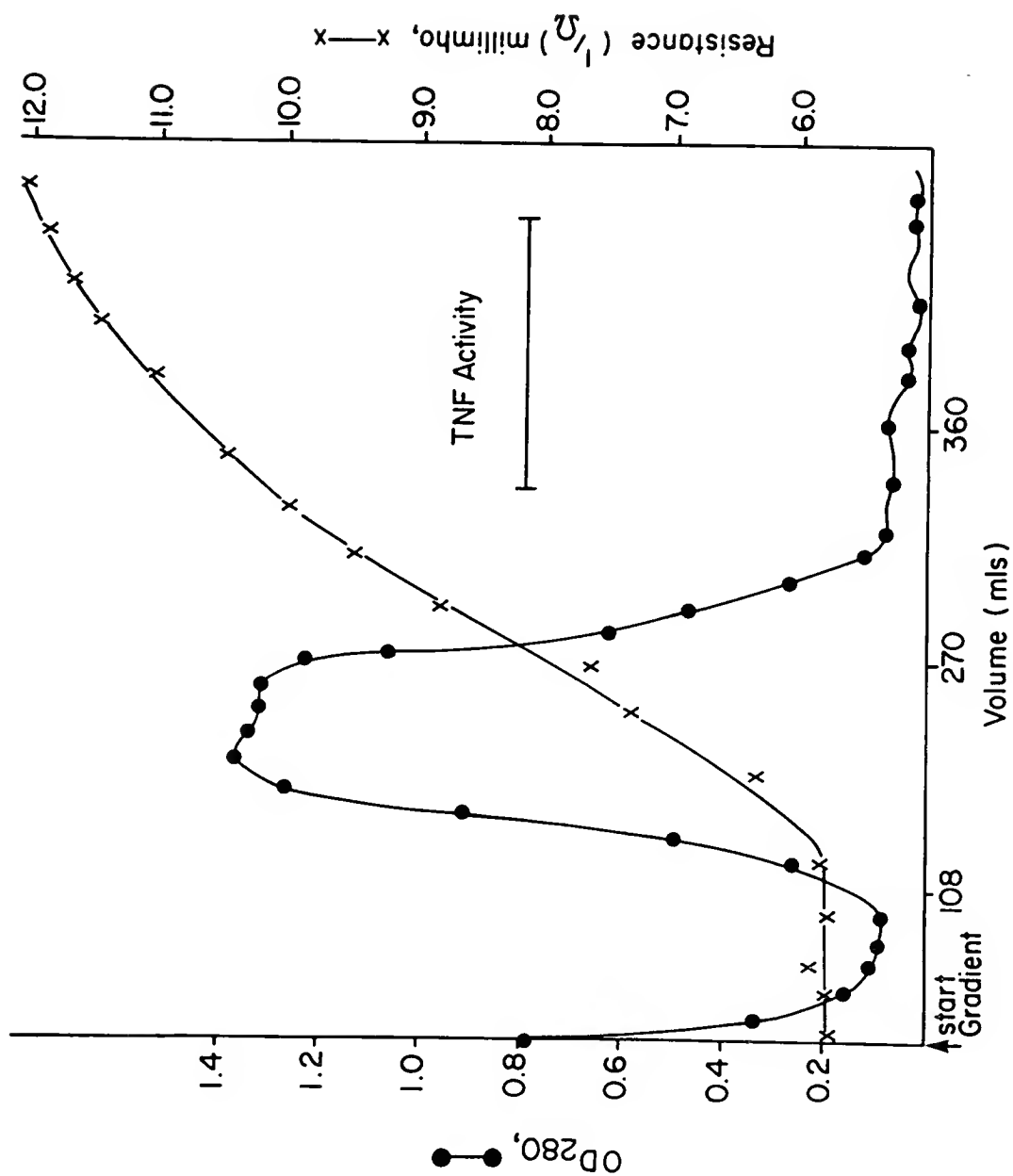
Not all transformed cells are affected by TNF. For example Old (1976) reported that 21 human tumor cell lines were screened for their sensitivity to TNF. TNF was cytostatic for 10, cytotoxic for 1, and had no effect on 10 others. The reason for the variable effects of TNF on transformed cells is presently unclear. TNF presumably recognizes common feature of some transformed cells, but not all, which is missing in normal cells.

### Purification and Physiochemical Properties of TNF

Since TNF is induced by a technique which causes a general reticuloendothelial hypertrophy one might expect TNF serum to be a rich source of lymphokines as well. Indeed this is the case. Interferon is produced in large quantities (Sauter and Gifford, 1966) as well as a factor which causes differentiation of B lymphocytes from complement receptor minus ( $CR^-$ ) to complement receptor positive ( $CR^+$ ) Hoffman et al., 1977). TNF serum also stimulated bone marrow progenitor cells

Figure 4. DEAE Sephadex chromatography. TNF containing samples from the preliminary gel filtration were loaded in starting buffer and unbound proteins were washed off the column. Elution was with a linear salt gradient from 120-400 mM NaCl. Indicated fraction with TNF activity were pooled and further purified by Con A Sepharose chromatography.





to differentiate to form colonies of macrophages or granulocytes in vitro (Shah et al., 1978; Butler et al., 1978). We realized that it would be necessary to purify it, free from other cell inhibitory and cytotoxicity activities.

Since we started with large volumes of rabbit serum we employed two preparative procedures as the first steps in purification of TNF, an ammonium sulfate precipitation and a gel filtration. Pilot studies indicate that less than 0.5% of TNF activity was present in the 60% saturated ammonium sulfate (SAS) supernatant fluid and that no appreciable increase in specific activity was provided by excluding a first precipitation with 20% SAS. The proteins precipitated in 60% ammonium sulfate were resuspended in 50 mM phosphate buffer, pH 7.0, and were then loaded onto an Ultragel Aca 34 column. Three major protein peaks were generated corresponding approximately to an excluded peak greater than 300,000 d, included proteins of range 40,000 d to 300,000 d and those less than 40,000 d. Fractions with TNF activity in the 40,000 to 300,000 d second peak were pooled. Gel filtration data for TNF is presented for a later purification step. Further resolution of TNF was then obtained by anion exchange chromatography on DEAE Sephadex (Fig 4). Unbound proteins were washed off the column with starting buffer and a linear NaCl gradient was used to elute TNF activity. Significant amounts of TNF began to elute in 50 mM phosphate buffer, pH 7.0, with a salt concentration of 160 mM and continued until approximately 260 mM NaCl. Shallower salt gradients over this range did not improve the resolution of this purification step. Fractions with TNF activity were then passed through a Con A affinity column. TNF did not bind to this column but several contaminating proteins were removed by this procedure. Since there was an appreciable loss of activity with this

step we verified that TNF did not exist as heterogeneously glycosylated species by performing pilot studies using DEAE Sephadex purified proteins with  $^{125}\text{I}$  labeled purified TNF added. TNF activity, as well as radioisotope counts, was only recovered in the wash through fractions. Sequential elution with  $\alpha$ -methyl-mannoside 20 mg/ml, 200 mM borate buffer, pH 6.9, or 3.0 M KCl did not release any activity or counts from the column. TNF activity is stable in borate and KCl, at least for the time required to make these determinations. The wash through fraction from the lectin column was therefore concentrated and loaded onto a Sephacryl-200 column for gel filtration. Glycerol was added to the buffer system to minimize protein adsorptive losses. As shown in Fig. 5, TNF activity was well resolved from the major contaminating proteins and gel filtered with a molecular weight of approximately 55,000 daltons. The specific activities resulting from the various purification steps are shown in Table II.

#### Criteria for Purification and Further Estimation of Molecular Weight

A portion of the TNF recovered from the Sephacryl-200 gel filtration column was iodinated and subjected to several analytical procedures. Labeled TNF was electrophoresed on 10% SDS containing polyacrylamide gels which were then dried and exposed for autoradiography. The homogeneity of the preparation was demonstrated by allowing films to overexpose in order to detect minor protein species (Fig. 6). We were not able to accurately compute the specific activity of TNF iodination since some cytochrome c, which was added as cold carrier, was also inadvertently labeled by unreacted Bolton-Hunter reagent. Approximately 230,000 total CPM of TNF plus carrier protein was loaded on this gel and film was exposed for 14 days. The apparent molecular

Figure 5. Gel filtration on Sephacryl-200. The wash through fractions from the Con A affinity column, which contained all the TNF activity, were concentrated and further purified by gel filtration. The column was calibrated with known molecular weight standards.

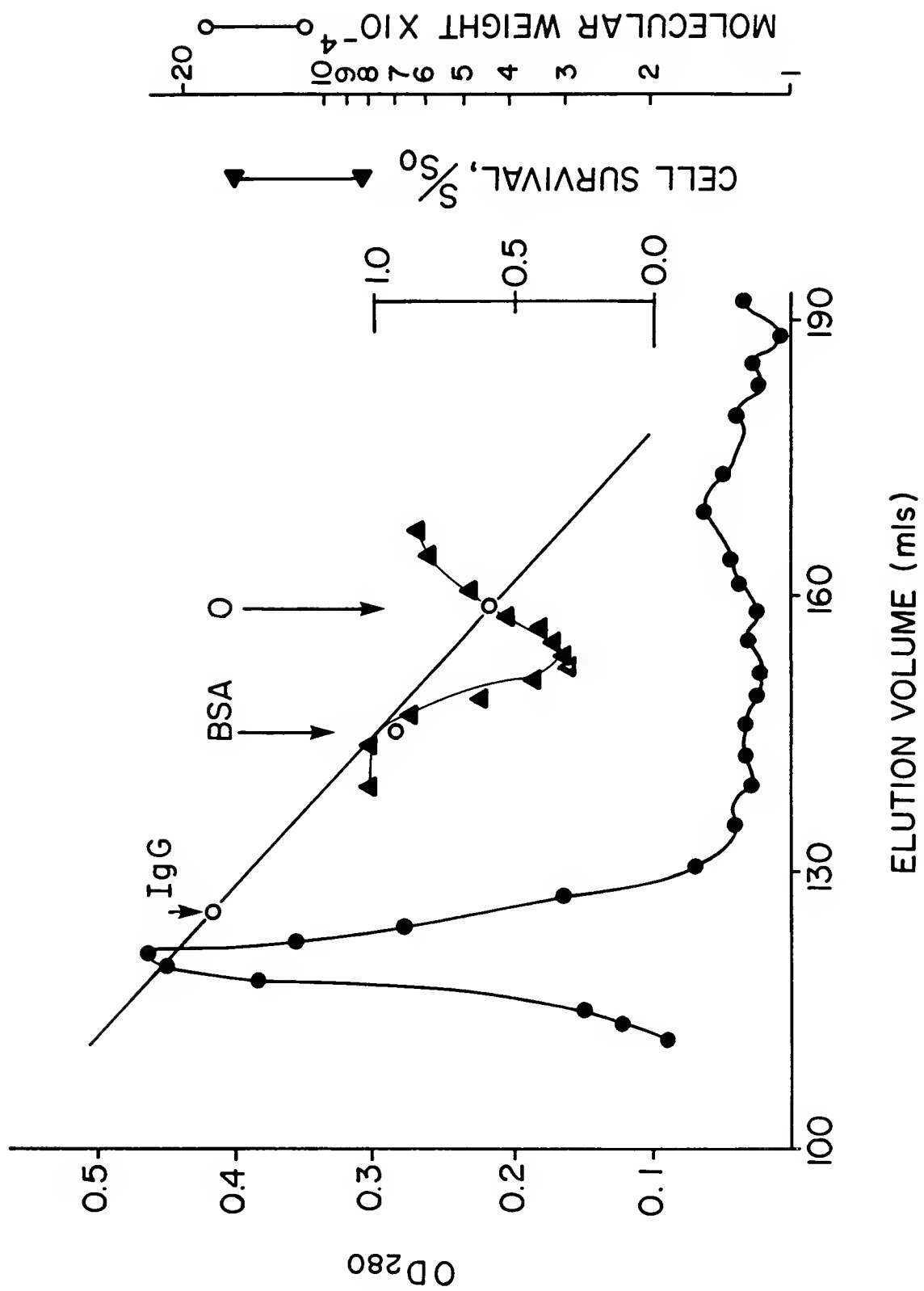


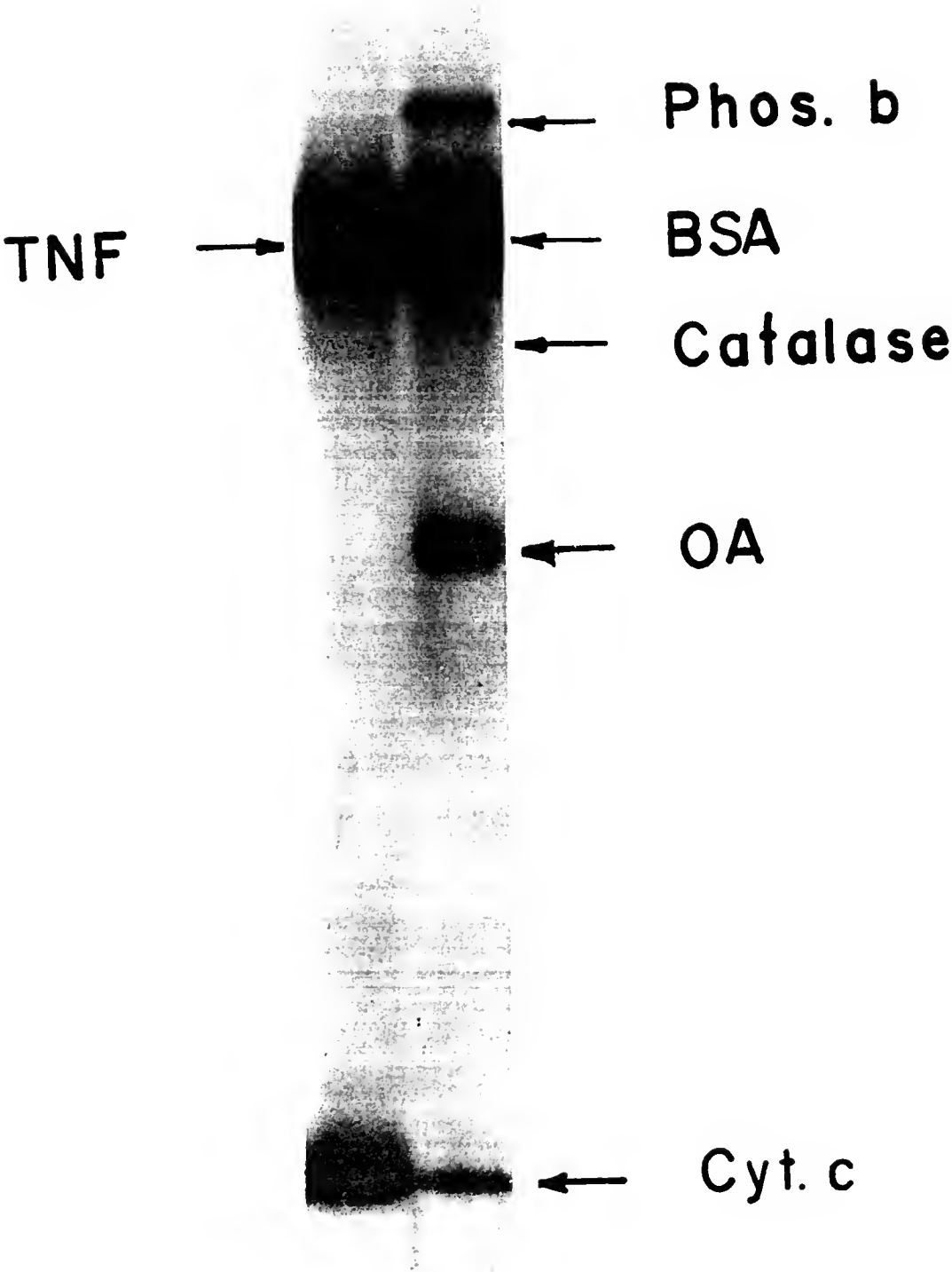
TABLE II

## Purification Summary

Sample	Volume (mls)	Protein (mg)	Units <sup>TNF<sup>a</sup></sup> ( $\times 10^{-3}$ ml <sup>-1</sup> )	Total Activity (U $\times 10^{-3}$ )	Specific Activity (U/mg $\times 10^{-3}$ )	Purifi- cation Factor	Recovery (Percent)
Crude Serum	100.0	50.0	7.5	750.0	0.15	1.00	100.0
60% SAS <sup>b</sup>							
Supernate	210.0	6.5	0.05	11.0	0.0075	0.05	0.014
Pellet	40.0	68.5	19.5	780.0	0.284	1.89	104.0
Ultragel Aca 34	250.0	3.3	2.5	625.0	0.76	5.06	83.0
DEAE Sephadex	15.0	4.77	24.0	360.0	5.03	33.5	48.0
Con A Sepharose Flow Through	2.0	4.97	41.5	83.0	8.35	56.0	11.1
$\alpha$ -MM Elution <sup>c</sup>	3.0	5.03	0.2	0.60	0.40	0.27	0.08
Sephacryl-200	0.6	0.368	115.0	69.0	312.0	2083.0	9.2

<sup>a</sup>Units are S50 as defined in the text.<sup>b</sup>SAS = Saturated ammonium sulfate<sup>c</sup> $\alpha$ -methylmannoside, 20 mg/ml

Figure 6. SDS-polyacrylamide gel electrophoresis Purified TNF and marker proteins were labeled by 125-iodination as detailed in Methods. Gels were dried and exposed for autoradiography for 14 days in order to reveal minor components. Markers were: phosphorylase b, bovine serum albumin, catalase, ovalbumin, and cytochrome c. Cytochrome c was added to purified TNF as carrier protein.





weight of purified TNF under these conditions was 68,000 d (Fig. 6). Purified TNF samples prepared for electrophoresis by boiling with SDS as well as the reducing agent  $\beta$ -mercaptoethanol showed an identical protein staining profile implying the absence of any reductive subunit structure for the molecule. (Data not shown.)

The electrophoretic characteristics of TNF were also examined under non-denaturing conditions. Partially purified TNF obtained from a cation exchange chromatographic step, CM Sepharose, was used in this analysis. As seen in Fig. 7 the TNF activity ran to the anodic side of the BSA marker, in the  $\alpha$ -globulin region of the gel. TNF activity was only associated with the single Coomassie R-250 staining band and is clearly resolved from the BSA marker. On two occasions 50-60% of the TNF activity could be recovered from the gel. Proteins recovered from the native gel were re-run on SDS-denaturing gels with various marker proteins. The major staining material co-migrated with the BSA marker, as it does in Fig. 6.

Since there was some discrepancy in the molecular weight evaluations for TNF between the gel filtration and SDS-PAGE we wished to ascertain the size characteristics of the molecule by a third method. Glycerol gradients were employed and TNF and marker proteins were run in parallel buckets. As can be seen in Fig. 8, TNF ran as a symmetrical peak with a molecular weight of 52,000 d, in agreement with the gel filtration data.

#### Isoelectric focusing

Purified  $^{125}\text{I}$  labeled and cold carrier TNF was subjected to isoelectric focusing. Replicate gels were either dried and prepared for autoradiography or sliced and assayed for TNF activity. Peak TNF activity was recovered in a fraction whose pH was 5.1 and which

Figure 7. Non-denaturing gel electrophoresis. Partially purified TNF was electrophoresed under native conditions as described. Gels were either stained for protein or cut into 8 mm slices, proteins eluted, and fractions assayed for TNF activity.

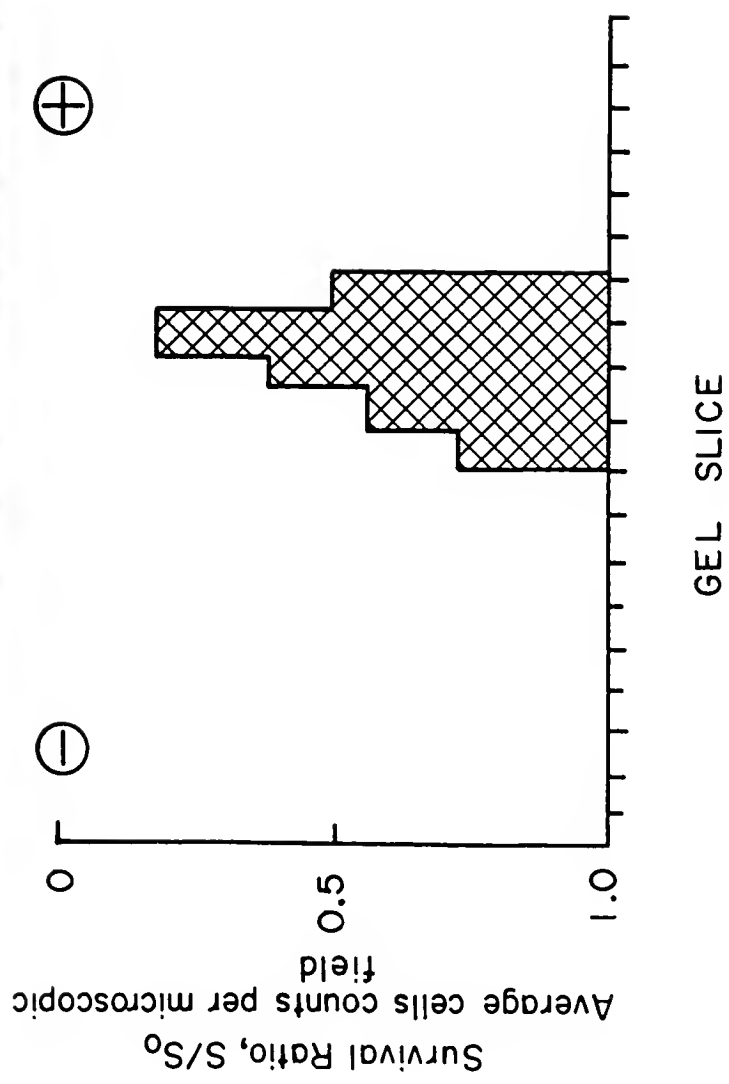
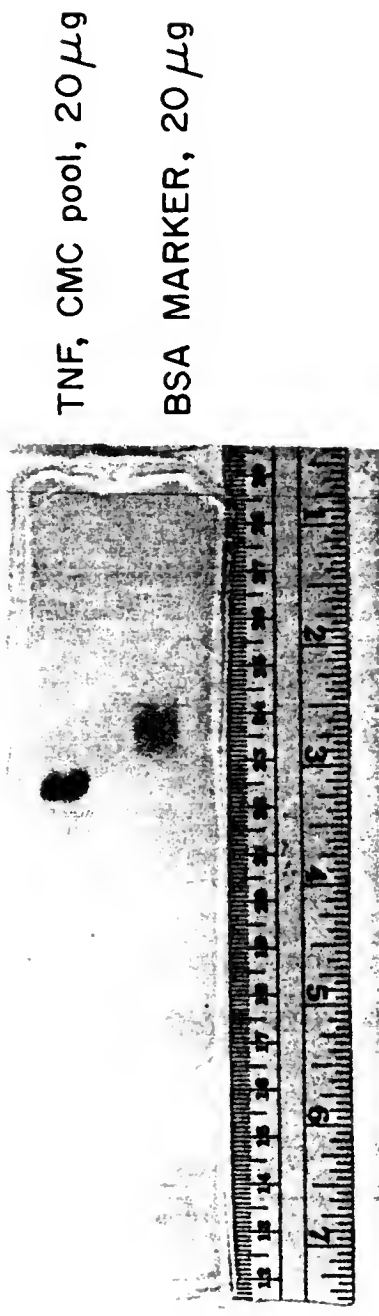
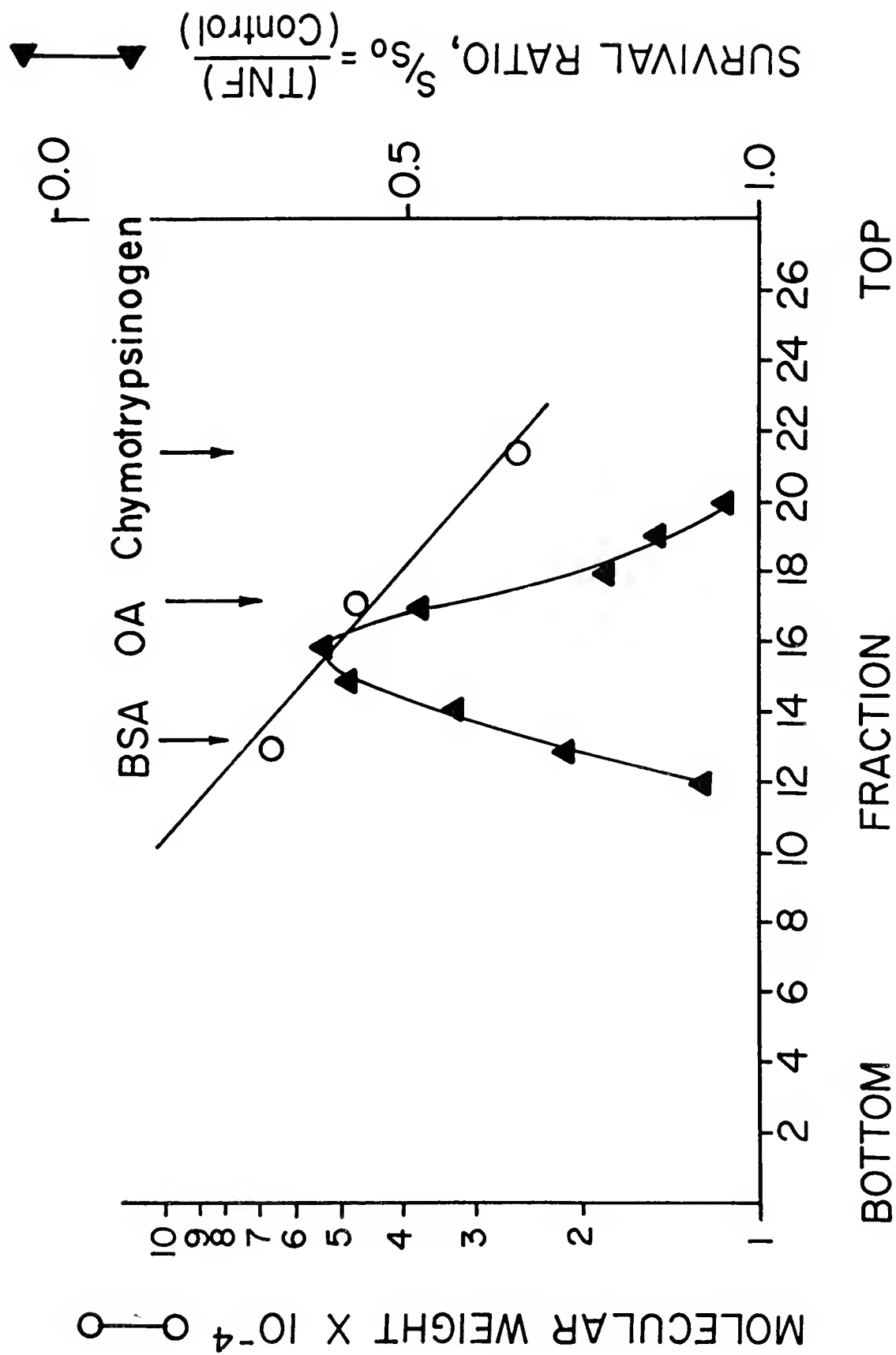


Figure 8. Glycerol gradient centrifugation. Purified TNF was sedimented through 20-40% glycerol gradients in Tris-NaCl buffer. Sedimentation was for 56 hours at 45,000 RPM at 4<sup>o</sup>. Indicated marker proteins were run in parallel buckets.



contained the single autoradiographic band (Fig. 9). Two dimensional isoelectric focusing and SDS-PAGE were also performed (Fig. 10) as described by Horst et al. (1979).

#### Enzyme digestions

The protein nature of TNF was demonstrated by its susceptibility to digestion by pronase (Table II). TNF was however relatively trypsin resistant, implying a low number of exposed basic amino acids. Extensive trypsin digestion did destroy TNF activity. TNF was also insensitive to neuraminidase digestion although there was a decrease in activity observed due to the pH 5.0 incubation. Phospholipase C also had no effect on TNF activity.

#### Sensitivity to pH

TNF activity was stable over the pH range of 6-10 (Fig. 11). The protein showed some loss of activity after exposure to pH 5 to 5.5 for two days at 4°C. At more acid pH the activity began to drop much more drastically, and the protein was completely inactivated at pH 3. In working with the protein we have found that activity will survive relatively brief (hours) exposure to pH levels as low as 4. However a 3 hour exposure at 37°, pH 5.0, resulted in a 60% loss of activity (Table III).

#### Temperature stability

Purified TNF in PBS, pH 7.0, survived heating at 70° for 1 hour with no loss in activity. Over 99.9% of the activity was however lost after 15 minutes at 80°.

#### Hemorrhagic necrosis by Rabbit TNF

The activity originally described by Carswell et al. (1975) as tumor necrosis factor had the following distinguishing characteristics. It was elicited in experimental animals following C. parvum or BCG

Figure 9. Isoelectric focusing. Purified TNF was focused as described. Gels were then sliced and assayed for pH and TNF activity.

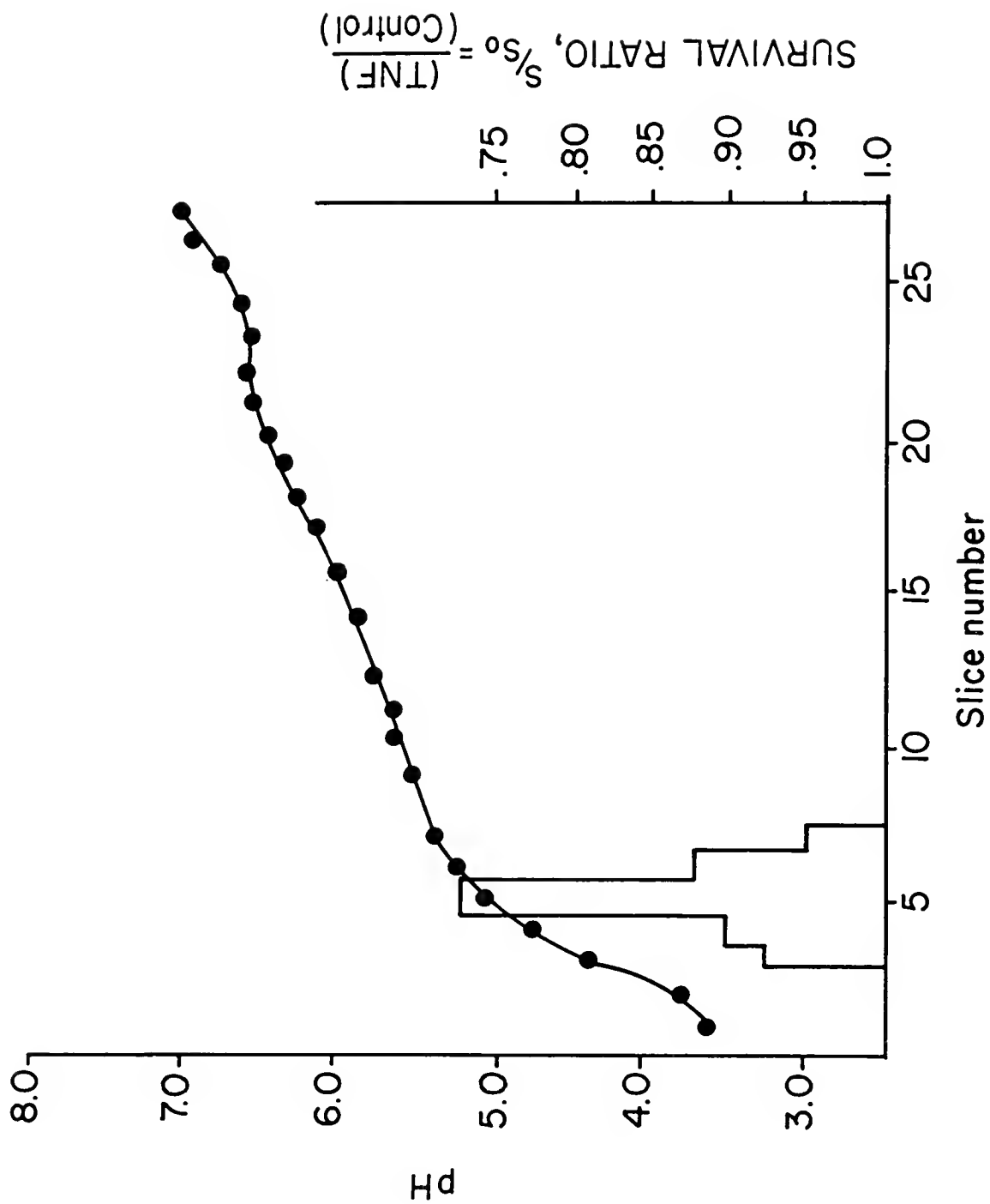




Figure 10. Two dimensional electrophoresis of purified TNF. TNF was iodinated as described. One major spot, corresponding to a molecular weight of 68K was observed. Ouchterlony immunoprecipitation indicated that this material was not serum albumin when tested against hyperimmune sheep anti-rabbit whole serum. A low level of reactivity to at least one other, non-identified, serum protein was observed.

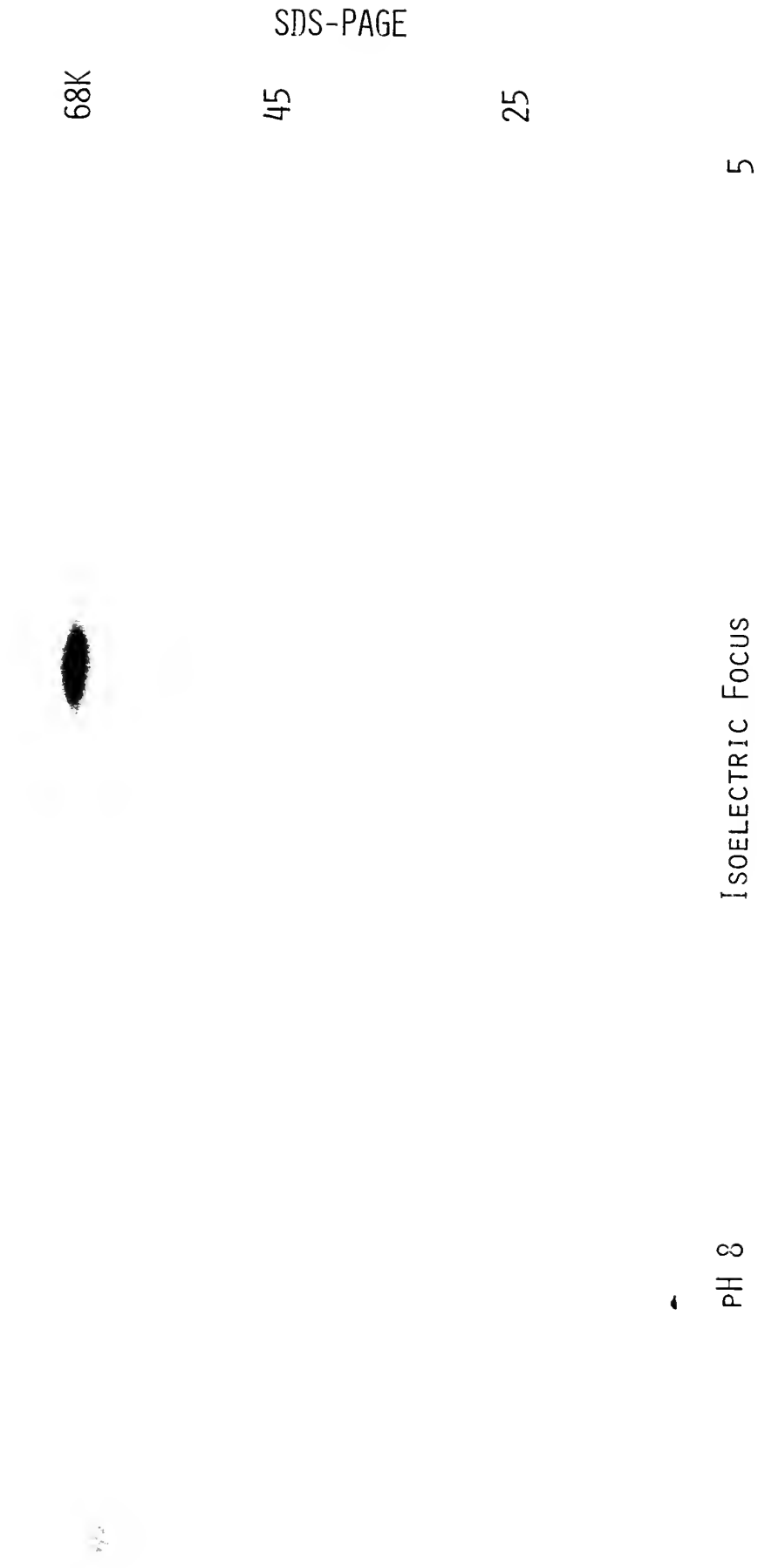


Figure 11. pH stability of TNF. Crude serum containing TNF activity was incubated at a 1:10 dilution in various pH buffers for 48 hours at 4° C. Samples were then diluted into pH 7 buffer and assayed for activity. Buffers used were: citrate-phosphate (2-6), phosphate (7), and glycine-NaOH (8-11).

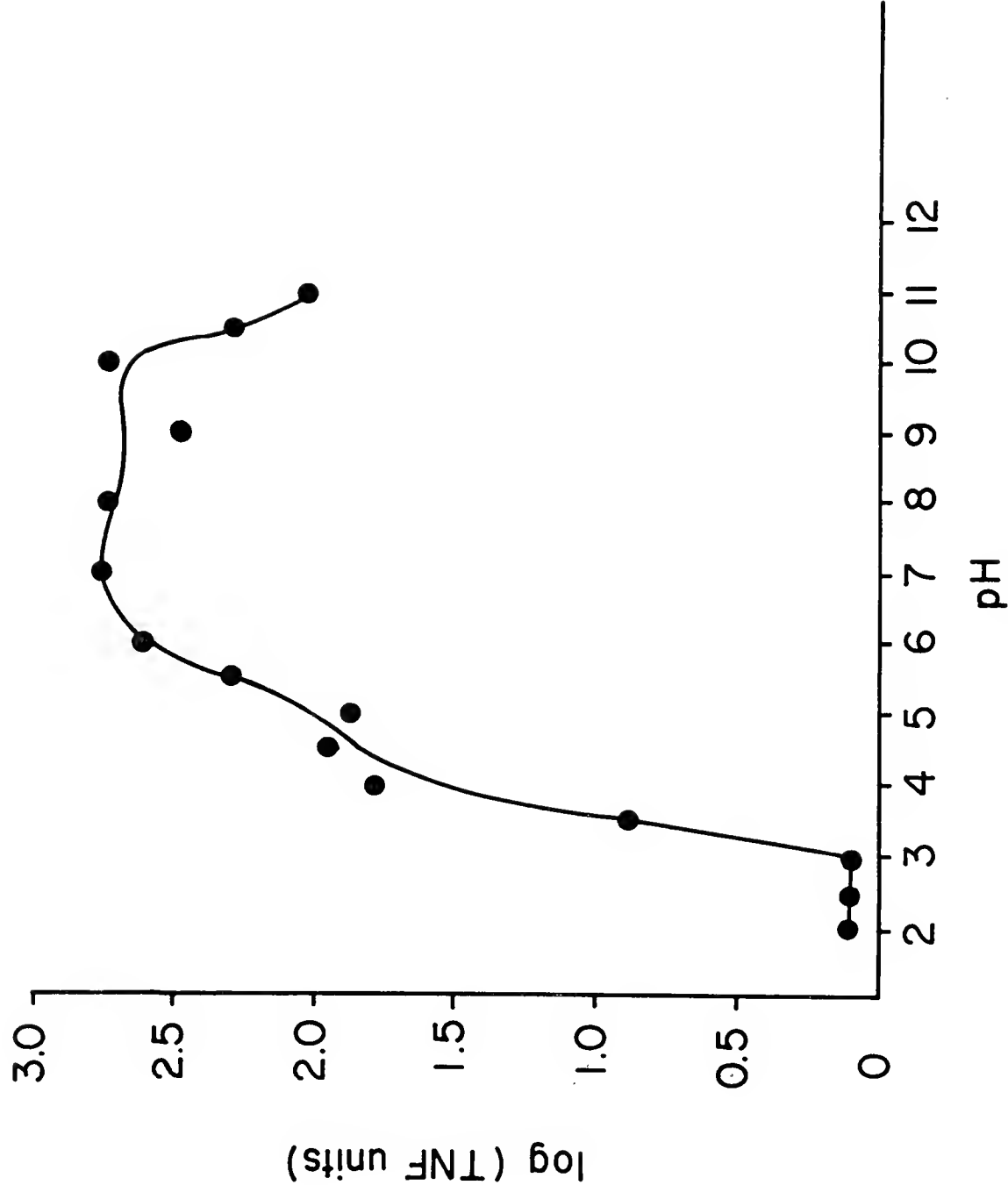


TABLE III

Stability of TNF to Digestion by Various Enzymes

Enzyme	Concentration μg	Activity Remaining <sup>a</sup>	
		Units (x 10 <sup>-3</sup> )	Percent of Control
Control (PBS)	-	116.0	100.0
Trypsin	25	123.0	106.0
Trypsin (32 hours)	250	1.8	2.0
Pronase	10	0.93	0.8
Phospholipase C	5	104.0	90.0
Control (pH 5 acetate buffer)	-	39.5 <sup>b</sup>	100.0
Neuraminidase	8	42.7	121.0

<sup>a</sup>Purified TNF, 25 μg, was incubated at 37° C with indicated enzymes for three hours as detailed in Methods. Extensive trypsin digestions were performed for 32 hours.

<sup>b</sup>Neuraminidase digestions were carried out at pH 5.0. TNF loses activity under these conditions (Figure 11); therefore a separate control was performed.

priming followed by endotoxin challenge, it was capable of selective toxicity for transformed compared to normal cells, and was capable of eliciting a necrotizing reaction within the transplanted solid tumor Meth A when passively administered to tumor bearing animals.

We employ the same procedure as Carswell et al. to produce crude TNF containing serum, and we have already reported on the discriminatory killing of transformed cells by this substance (Ostrove and Gifford, 1979; Gifford et al., 1980; Ruff and Gifford, 1980a; Ruff and Gifford, 1980b). In order to evaluate whether rabbit TNF, and in particular whether purified rabbit TNF was capable of eliciting hemorrhagic necrosis, tumor bearing animals were tested with control serum, crude TNF serum, and partially purified TNF. Purified TNF was not available in sufficient amounts for this test. Control serum did not elicit a necrotic reaction within the tumor mass. Crude and partially purified TNF elicited a 1<sup>+</sup> hemorrhagic reaction indicating that the designation of TNF for this protein activity is correct. A list of the physical-chemical properties of purified rabbit and partially purified mouse TNF is included in Table IV.

#### Mechanisms of Action and Cellular Effects

The following discussion will describe our initial attempts to gain an insight of the general kinetic parameters as well as the range of effects which TNF has on cells. Many of the studies were performed with crude TNF while we were awaiting final purification. They are therefore subject to certain reservations, primarily a concern that effects which we observe may have some contribution from contaminating activities present in TNF containing serum. Where results have been verified with purified preparations they will be so indicated.

TABLE IV  
Physio-Chemical Characteristics of TNF

	Rabbit	Mouse
<u>Molecular Weight</u>		
Gel Filtration	52K 40-50K <sup>b</sup> -	150K <sup>a</sup> 125K <sup>c</sup> 60K high salt <sup>c</sup>
Glycerol Gradient	50K	-
SDS PAGE	68K <sub>b</sub> 60K	- -
<u>Non-denaturing Electro-</u> <u>phoresis</u>		
	$\alpha$ -globulin	$\alpha$ -globulin <sup>a</sup>
<u>pH Stability</u>	5-10 stable	-
<u>Isoelectric Point</u>	5.1	-
<u>Temperature Stability</u>		
	70 C, 1 hr. stable 80 C, 15 min. unstable	56 C stable <sup>d</sup> 70 C, 1 hr. unstable <sup>a</sup>
<u>Denaturing Agents</u>		
	Acetone, 2-mercapto- ethanol	-
<u>Enzyme Sensitivities</u>		
	Neuraminidase-stable	-
	Trypsin-sensitive	-
	Pronase-sensitive	-
	Phospholipase c-stable	-
<u>Glycoprotein</u>		
	Presumably not	Presumably yes, sialic acid galactosamine

<sup>a</sup>Green et al. (1976)

<sup>b</sup>Matthews and Watkins (1978)

<sup>c</sup>Mannel (1979a)

<sup>d</sup>Carswell et al. (197 )

### Dose Kinetics

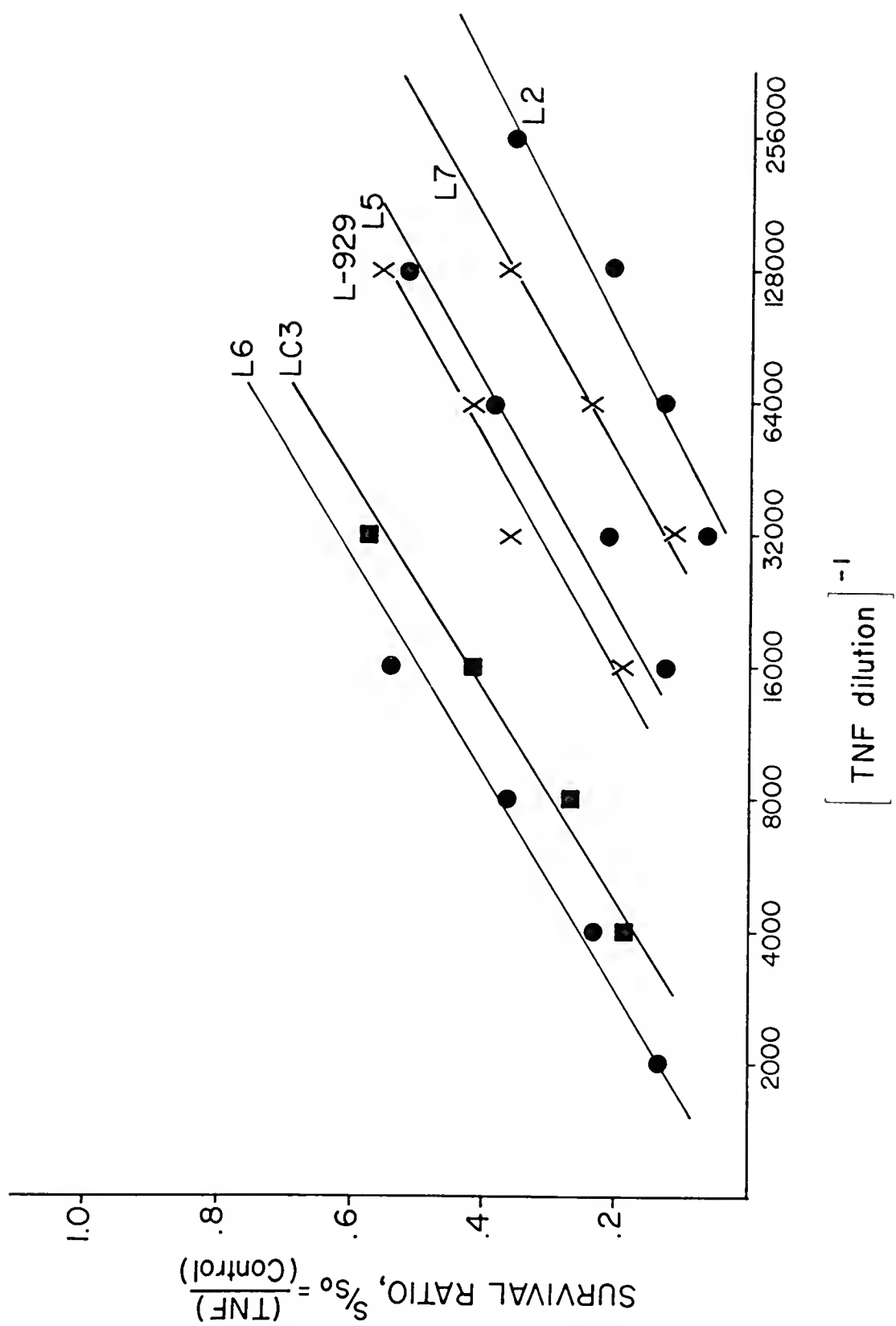
The shape of the dose response curve (Figs. 1-3 ) has some features worth noting. The curve is approximately sigmoidal when the abscissa is plotted on a logarithmic scale. A common interpretation is that the decrease in slope at high concentrations of TNF is due to redundant sites of injury, while the decreased slope at low concentrations is, in general terms, due to insufficient events to complete cell killing. The slope of the dose curve can be expressed as the fractional change in cell numbers compared to the log decrease in dose, measured over the linear portion of the curve. Theoretical considerations (Gifford and Koch, 1969) show that a slope of 0.705 would be expected for single-hit kinetics, i.e. one molecule of TNF can kill a cell. Since TNF dose curves consistently have slopes much less than 0.705, in the range of 0.35 - 0.45, one interpretation is that one molecule of TNF can kill more than one cell. This is compatible with an enzymatic mode of action for TNF, although other interpretations are possible.

Poisson statistics are appropriately applied to homogeneous populations and in their theoretical considerations Gifford and Koch (1969) point out that variation in sensitivities among members of a population may lead to artificially reduced slope. For example, they show that if two types of cells exist, differing only in their sensitivity to a particular agent such that they have identical probit regressions, but displaced along the abscissa relative to one another, and both curves have slopes of 0.705, then an equal mixture of the two populations would generate an intermediate curve with slope of 0.56 (i.e., low slope).

In order to evaluate possible heterogeneity of cell types in the population of L-929 cells with respect to sensitivity to TNF killing we prepared clones of L-929 cells. Figure 12 shows the dose relations for



Figure 12. The effect of TNF dose on the killing of various clones of L-929 cells.



five clones which we surveyed. We readily isolated clonal lines with varied sensitivity to TNF. Interestingly however all the clones have dose regressions with slope identical to the parental population. Thus the "low slope" of the dose curve we obtain in the bulk population is not due to the summation of "high slopes" of individual population members. The similarity in slope among clones and parental population was an unexpected observation since in the hypothetical case where there is a 1:1 mixture of two cells with varying sensitivities to a toxic agent the net toxicity as a function of dose would not be expected to generate parallel curves. For example, at low concentrations of the toxic agent the contribution to numbers of dead cells by the less sensitive population would be negligible compared to the more sensitive cells. The total mortality will be almost identical to that for the sensitive cell alone. With increasing cytotoxin concentration the contribution from the less sensitive population begins to be observed so that now the total mortality is greater than for the sensitive cell alone. Finney (1964) discusses these conditions and further points out that for the case where the dosage regression lines for mixtures of cells and for the constituent cells are all parallel a synergistic mechanism may be operative. That is to say a more TNF resistant cell might tend to "protect" a more sensitive cell, or the converse.

## Time Kinetics

### Onset and rate of cell killing

Growth curves for L-929 cells cultured in the continuous presence of various amounts of TNF (Fig. 13) reveal a time and concentration dependence for cell killing. The onset of cell death, measured by counting intact cells microscopically, is delayed for approximately 10 hours, even in the presence of relatively high doses of TNF. When, in the same experiment, sensitive target cells are isotopically pre-labeled with  $^3\text{H}$ -thymidine, one finds that high doses of TNF are more rapidly cytotoxic for these cells, as indicated by the 63% release of label at 12 hours (Fig 14). The membrane permeability change precedes the more slowly progressing overt cell destruction since cell recovery did not begin to drop substantially for an additional 12 hours. More dilute amounts of TNF result in a dose and time dependent decrease in isotope release and growth inhibition. Thus increasing amounts of TNF result in cytostasis, followed by leakage of labeled macromolecules or degradation products, followed finally by complete cell disruption. These data also indicated that both proliferation inhibition as well as cytolysis occur in TNF treated cultures.

The time kinetics of cell killing due to TNF was examined in more detail by two methods. In a cinematographic study, time-lapse photography has provided a more detailed analysis of the rate of cell killing, as well as the apparent lag period (Fig. 15). In the case where one deals with larger populations of cells, it is not possible to exclude the possibility that a small fraction of cells may be dying at times significantly earlier than 10-12 hours. The absence or presence of a lag period, as well as its duration must be incorporated into any mechanistic theory of TNF action. The time lapse photography provides

Figure 13. Growth curves for L-929 cells cultured in the presence of various doses of TNF or normal serum.

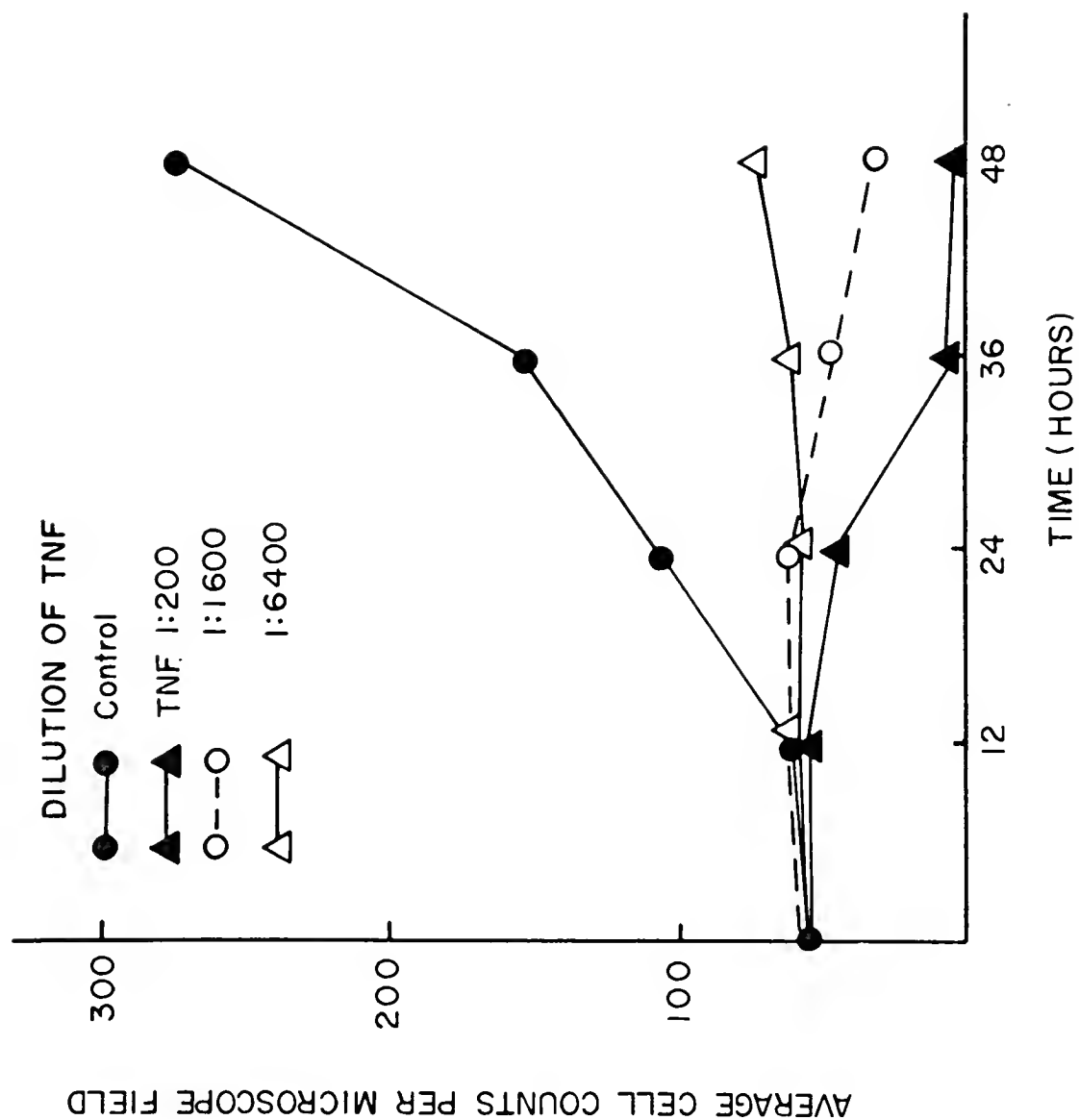


Figure 14. Time course of L-929 cell killing in the same experiment as Figure 13, determined by the cytotoxic release of isotope from cells which had been pre-labeled with  $^3\text{H}$ -thymidine.

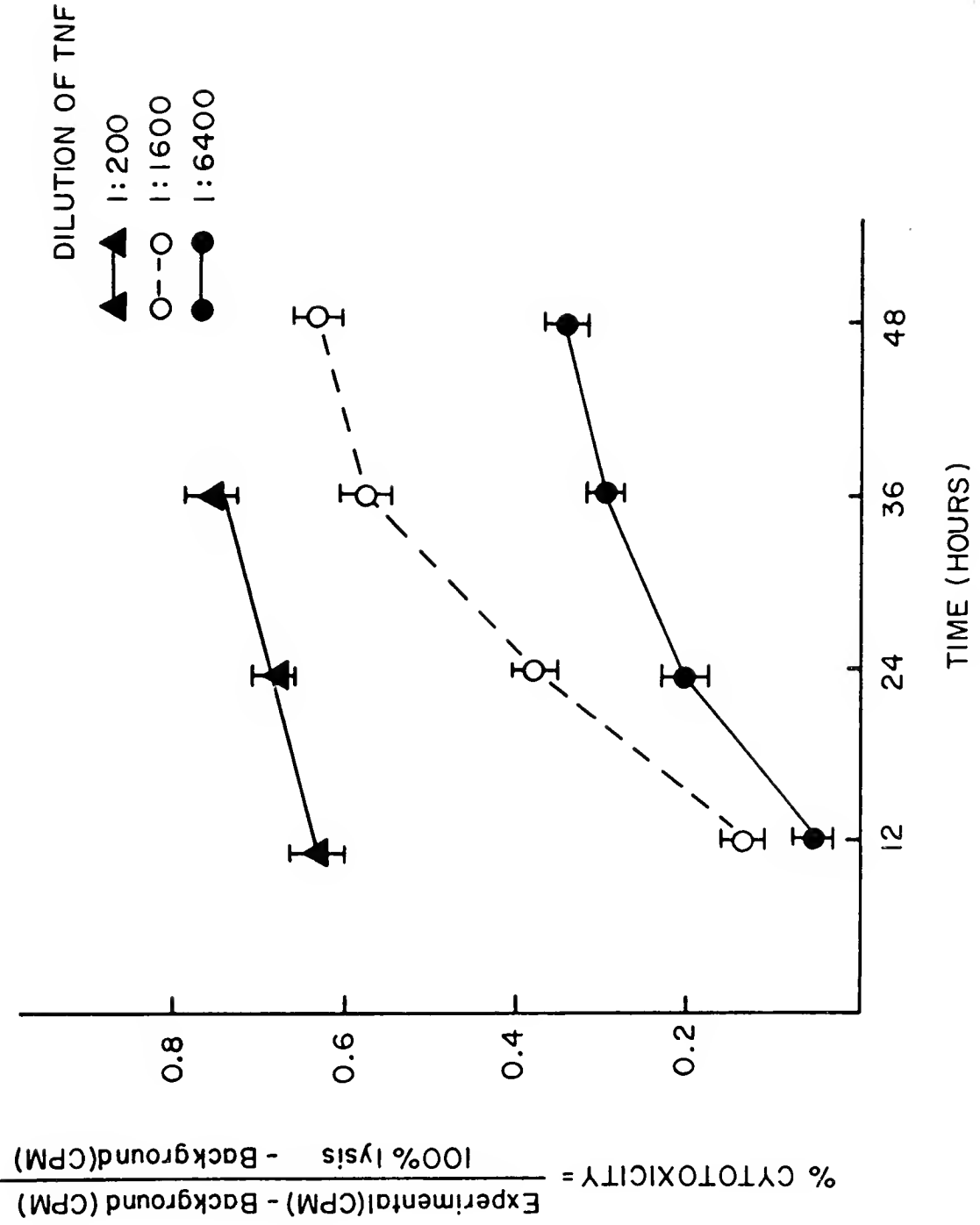
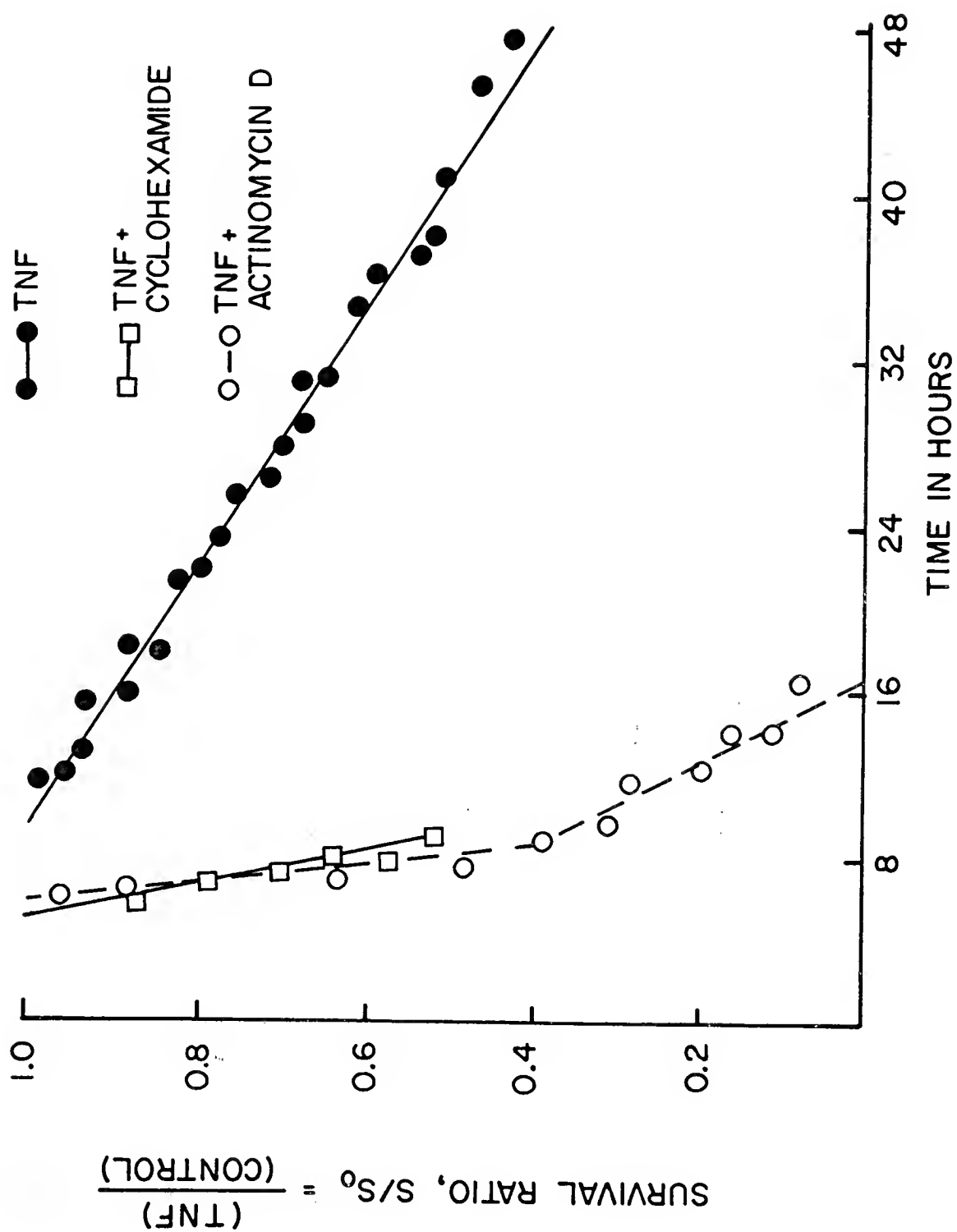




Figure 15. Time course of TNF killing in the presence or absence of actinomycin D or cycloheximide. Time lapse cinematography was employed (1 frame/15 seconds) and later analyzed for the time of cell death.



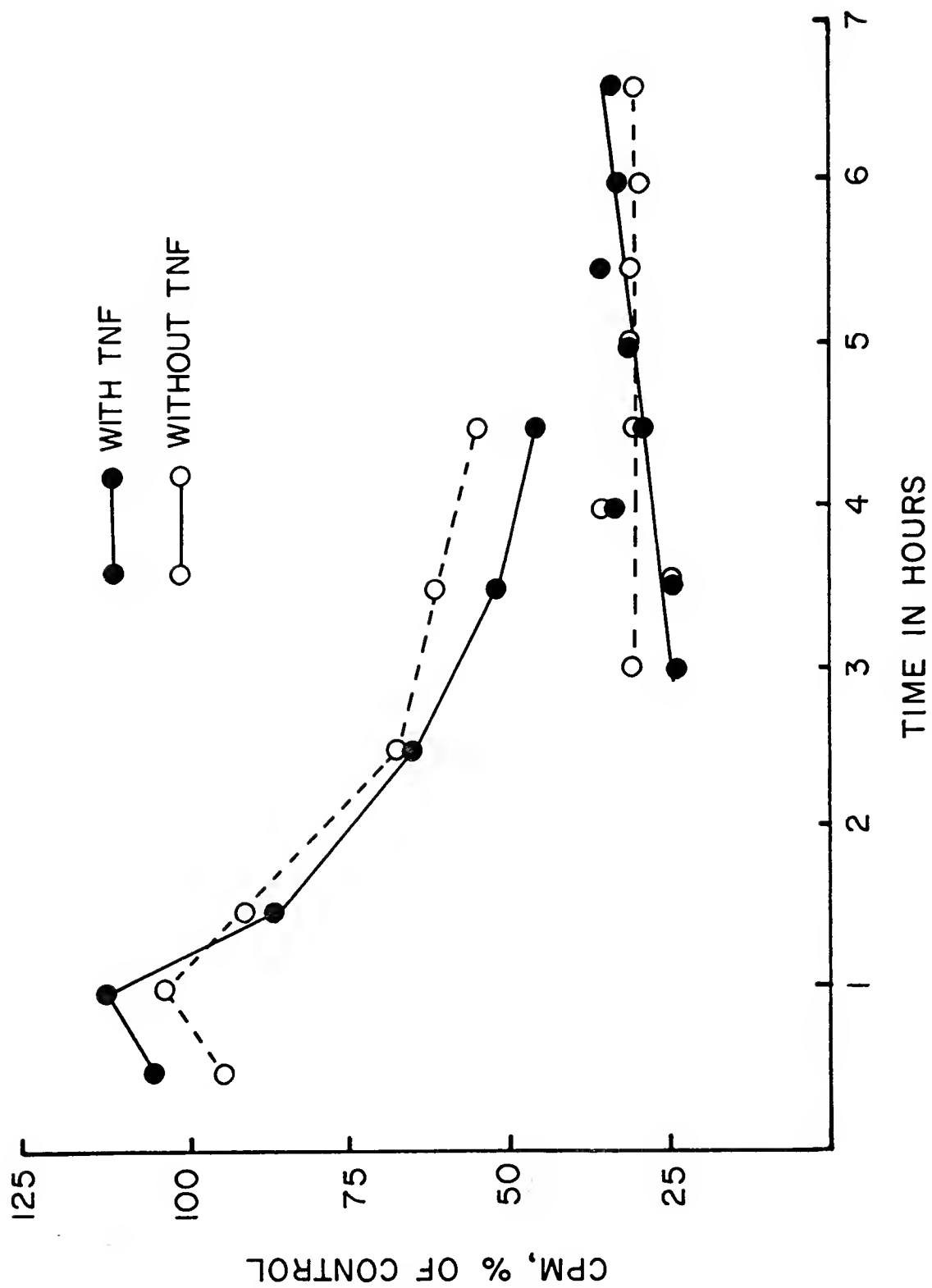
the capability to observe single cells for the duration of the experiment. Just before the cells die they start to round up and become very granular, a process that takes about 15 minutes (real time). At the moment of membrane depolarization the cells seem to "pop" and immediately become very refractile. This event was used to mark cell death. A plot of survival ratio  $S/S_0$  vs time of individual cell death generated a first order decay curve with a lag period of 10 hours, the time the first cell died, in agreement with Fig 13. The slope of this curve corresponds to a rate of cell killing of 1.6 percent/hr.

#### Effects of dose on cell killing

The effect of different doses of TNF on the onset of cell death and rate of cell death were most conveniently determined by staining trays of L cells at various times and determining survival ratios photometrically. We have already referred to the synergistic killing effects of TNF in the presence of actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis, respectively. Since cells which were treated with these inhibitors were blocked from dividing, it was possible to determine the kinetic parameters of cell killing independent of a background of cell division.

Actinomycin D, at 1  $\mu\text{g/ml}$ , enhanced the rate of cell killing by 10 fold compared to its non-inhibitor treated control. Additionally, the lag period was shortened from 10 hours to 4 hours (Fig. 15). Cycloheximide, at 5  $\mu\text{g/ml}$ , showed identical results. Since cycloheximide blocked cells did not die any sooner or faster than the actinomycin D blocked cells, this would imply that essential new translation for prolonged survival does not detectably lag behind new transcription. Cells pretreated with actinomycin D for four hours prior to TNF

Figure 16. Effect of TNF on the functioning of mRNA. The ability of cells to incorporate  $^3\text{H}$ -isoleucine into acid insoluble material at various times after the addition of actinomycin D. TNF was added either simultaneously with actinomycin D (curves starting at 0 time) or after 3 hours prior exposure to actinomycin D (curves starting at 3 hours). At the indicated times cells were pulsed with isotope for 30 minutes and TCA precipitable counts determined.



addition showed identical kinetics to non-pretreated cells. One possible mechanism of action that we considered was that TNF may have accelerated the normal decay of mRNA or that it inhibited mRNA function. Such a mechanism would help explain why both cycloheximide and actinomycin D treatment of target cells resulted in a similar accelerated kinetics of cell killing by TNF. TNF and actinomycin D were added to L cell cultures and at various periods of time thereafter replicate wells were washed with isoleucine-free medium and then isoleucine-free medium with actinomycin D was added and the cultures pulsed with  $^3\text{H}$ -isoleucine for 30 minutes. Incorporation of  $^3\text{H}$ -isoleucine into trichloroacetic acid insoluble material was then determined. As shown in Fig. 16, the loss of mRNA activity as a function of time was not significantly influenced by TNF. We did not determine any incorporation studies beyond 4 1/2 hours since significant cell killing begins at that time. Since pre-treatment with actinomycin D for 3 to 4 hours prior to TNF addition does not result in any further enhancement of cell killing, it was possible that TNF might inhibit a more stable mRNA species. Cultures were pre-incubated with actinomycin D for 3 hours and then TNF added. As can be seen in the figure, TNF has no effect on the ability of the stable forms of the messenger to be translated.

We also determined the effect of addition of cyclohexamide and TNF on the possible hydrolysis and/or release of prelabelled protein at various periods of time after the addition of TNF. Cells were prelabelled for 2 hours with  $^3\text{H}$ -isoleucine, washed to remove unincorporated label and incubated for an additional 1 1/2 hours to chase the label into protein. Following washing again, medium with cycloheximide, with or without TNF was then added. The amount of label in the

supernatant fluid (acid soluble and acid insoluble) was measured as a function of time. The amount of label found in the acid insoluble material is shown in Fig. 17. Significant increase in protein released from TNF treated cells appeared between 5 and 6 hours after TNF treatment. The enhancement of isotope release between 5 and 6 hours is consistent with the accelerated killing kinetics by TNF in the presence of cycloheximide or actinomycin D. Significantly, no differences in acid soluble extracellular label appeared, indicating that widespread and general protein hydrolysis was not the cause of TNF induced cytolysis. Within the context of the proposed repair capacity of the target cells (Ostrove and Gifford, 1979) these data would be consistent with the following. At a very preliminary level the enhancement of cell killing by TNF in the presence of inhibitors of protein synthesis would suggest a protein target for TNF. A corollary argument is that TNF does not merely block protein synthesis but must inactivate or destroy a protein dependent cellular function, perhaps structural. Because sensitivity to cycloheximide and actinomycin D show similar kinetics of killing, which is not enhanced by pre-treatment with inhibitors, the putative "repair" mRNA species is likely not to be constitutively expressed but rather induced by TNF treatment and co-ordinately translated.

Some additional features of cell killing are worth noting at this time. The lag period which precedes cytolysis is dependent on TNF concentration, more dilute amounts of TNF extend the lag period. As noted earlier there appears to be a minimal time of approximately 4-5 hours in which no cell killing occurs. The dependence of the lag on TNF dose shows higher than first order kinetics (Fig. 18). A linear transformation of these data can be arrived at by plotting lag (hours) vs. log

Figure 17. Effect of TNF on the release of acid insoluble counts from cells prelabeled with  $^3\text{H}$ -isoleucine. Cycloheximide was added at the same time as TNF and supernatants were precipitated with TCA and counts in the pellets were determined at the indicated times.



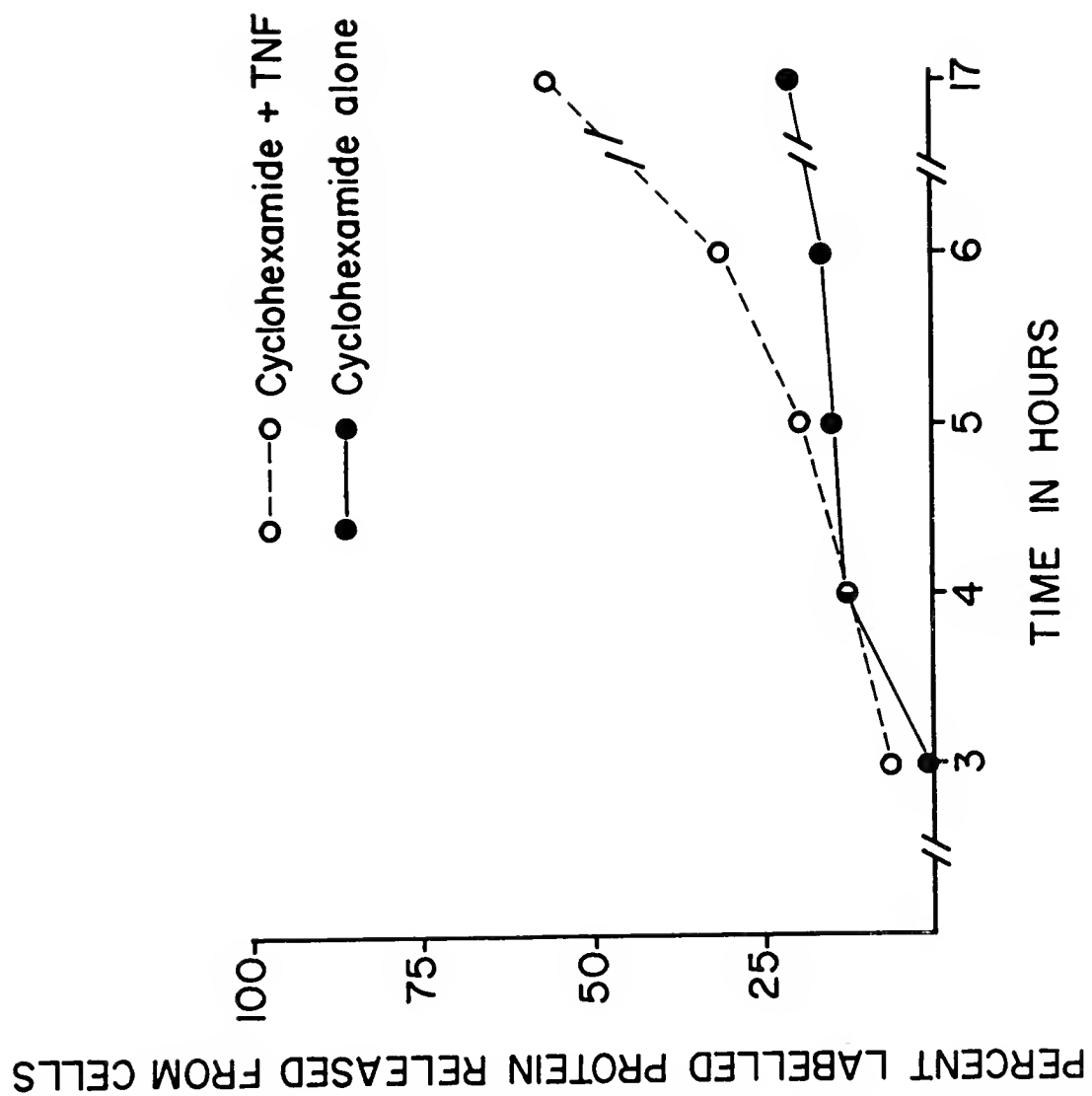
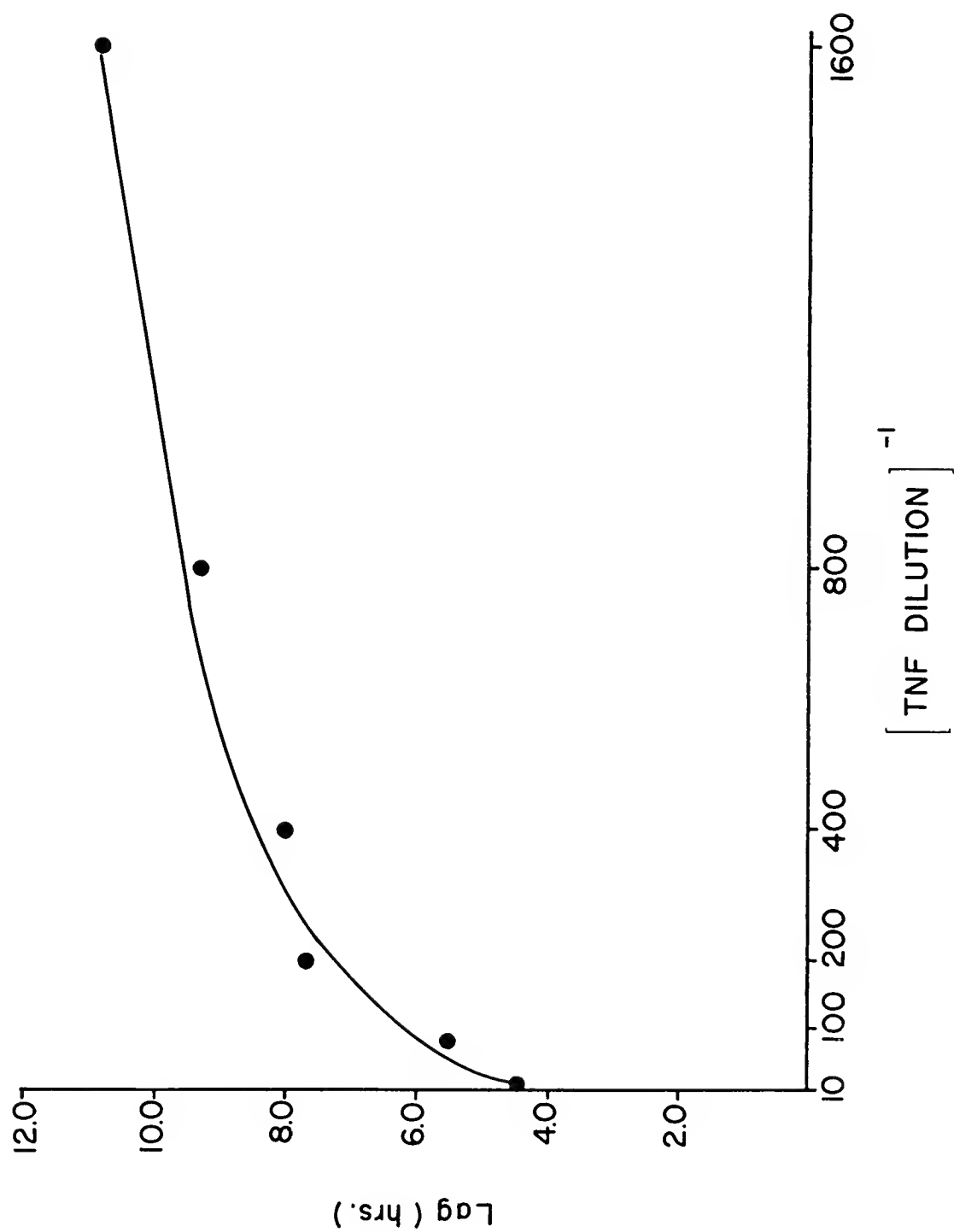


Figure 18. Effect of TNF concentration on the length of the lag period which precedes the onset of cytolysis. Data are derived from Figure 20.



(TNF dose) (Fig. 19). An interpretation would be that the onset of cell death, and by extension the killing process itself, is not dependent solely on the concentration of TNF but rather, on a simplistic level, there must be an additional component in the system. One speculation, consistent with the observation, would be that TNF has to gain entrance to the intracellular compartment to express its effect. The lag represents the minimum time period required for the cell to achieve a steady state concentration of TNF incompatible with continued viability. In a later section the inhibition of TNF action by agents which disrupt microtubules and microfilaments will be argued as also suggesting internalization of TNF, as opposed to action strictly in the external environment, such as at the cell surface.

The rate at which cells die, however, seems to be independent of TNF concentration over the 160 fold range examined (Fig. 20). Once the threshold limits which commit a cell to destruction have been reached further insult does not cause it to die any faster. Serum TNF may therefore not be the immediate agent of cell death. The actual cytotoxic mediator may be "activated" TNF or cellular hydrolytic enzymes. One alternative explanation would be that as far as the actual killing event is concerned we are still working in a range of TNF excess so that the system, even at lower doses of TNF, is effectively saturated. These type of studies provide useful guidelines for further work but they suffer from an important flaw in the sense that a study of kinetics of cell killing does not directly provide information on the

Figure 19. Logarithmic transformation of the data presented in Figure 18.

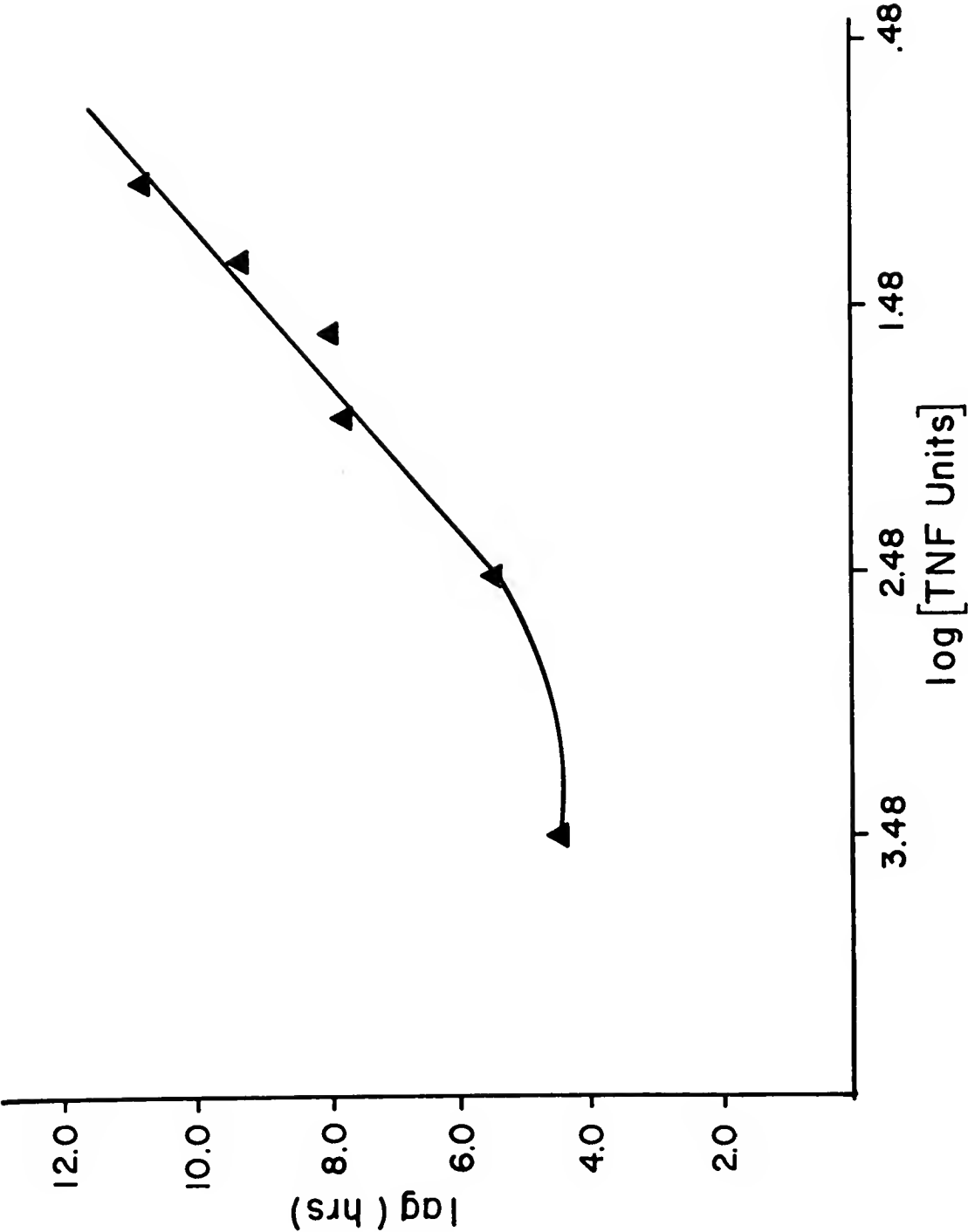
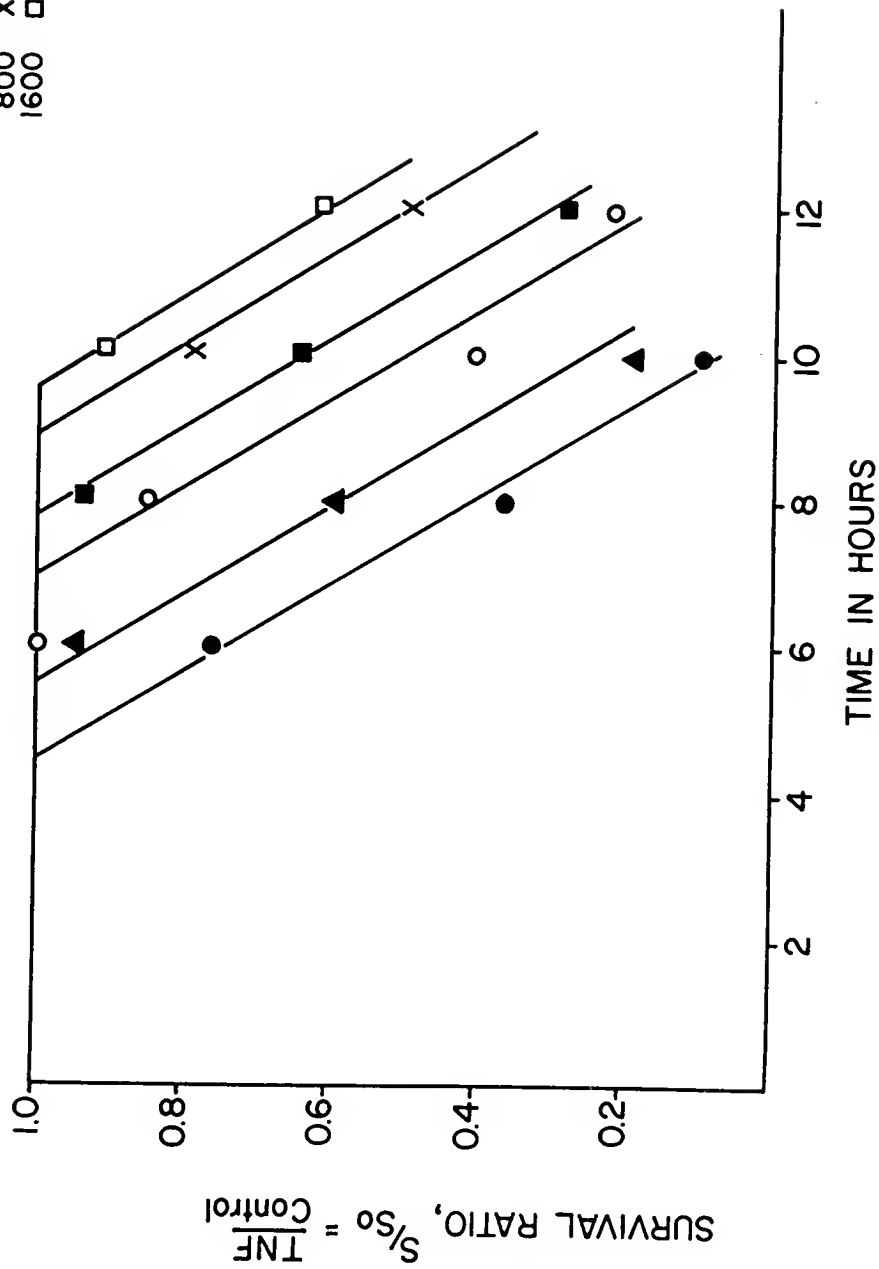


Figure 20. Effect of TNF concentration on the killing kinetics of L-929 cells. Cells were cultured in the presence of actinomycin D and various concentrations of TNF. Survival ratios were determined as a function of time.

DILUTION OF TNF

- 1:10
- ▲ 100
- 200
- 400
- X 800
- 1600





kinetics of TNF action. Cell death must necessarily be the last of perhaps many intervening events and must also be the converging point for any substance which kills a cell. Further work to elucidate the mechanisms of any cytotoxin must focus on the earliest detectable alterations within the cell.

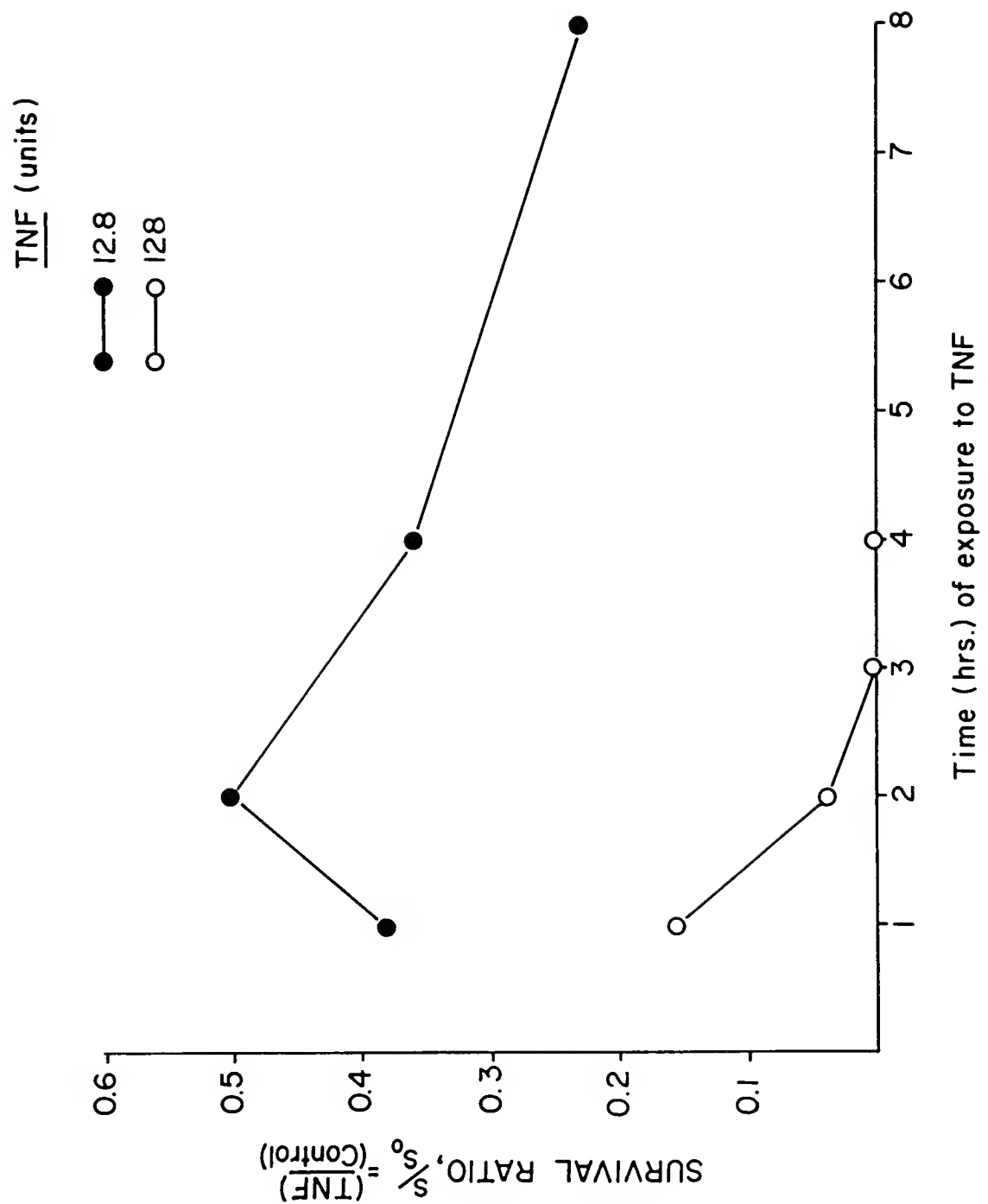
#### Killing by limited exposure to TNF

We wondered how long TNF had to be present on cells to exert its killing effects. In the absence of any inhibitors, cells were treated with pulses of TNF for increasing periods of time. TNF was removed by washing and cultures were re-fed in TNF free medium. Survival ratios were determined at the end of 48 hours. A period of exposure of 1 hour with a high dose of TNF is sufficient to result in appreciable mortality at the end of 48 hours (Fig. 21). Pulses with ten fold lower concentrations of TNF serum also show appreciable killing after limited exposure to the cytotoxin. Cells thus seem to be committed to die within the first hour and the continuous presence of TNF is not required.

#### Temperature dependence of TNF action

Matthews and Watkins (1978) reported that L-929 cells grown in the continuous presence of TNF at 21° were not killed at the end of four days by a dose of TNF which killed most cells in 2 days at 37°. Furthermore we have ascertained that such temperature inhibited cells, at the end of four days, when shifted up to 37°, promptly all die within the next 24 hrs. Although control 37° cultures do show some lethality at 24 hrs (fig. 13), the acceleration of killing kinetics after pre-treatment for several days at 25° suggests that there may be both temperature dependent and independent aspects of TNF action.

Figure 21. L-929 targets were treated with high concentrations of TNF for various periods of time. The cultures were washed and re-fed in the absence of TNF. Cell killing was evaluated at the end of 48 hours by the micromorphological method. 12.8 units of TNF corresponds to a 1:10 dilution of crude serum or 4  $\mu\text{g/ml}$ .

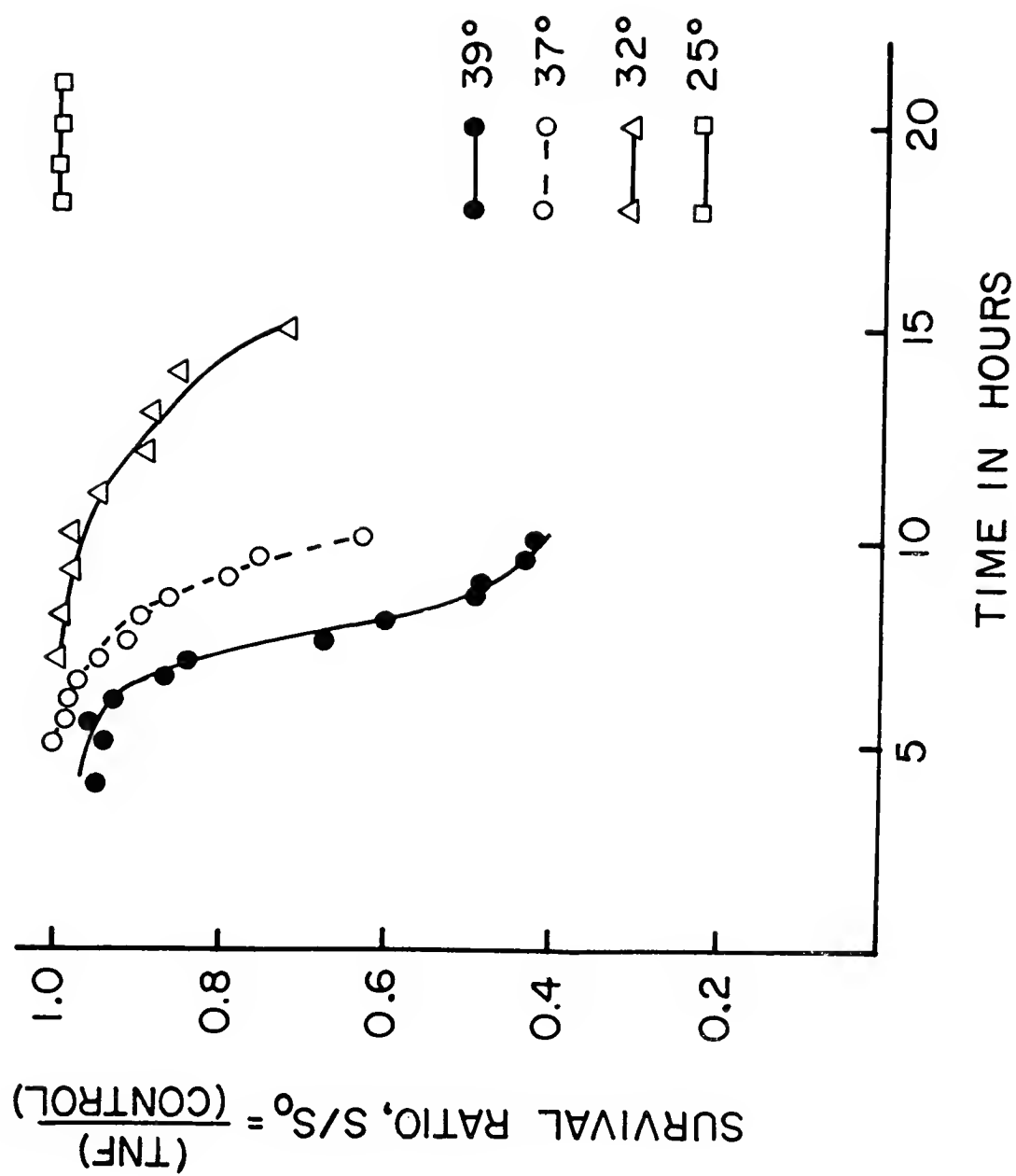


A more extensive survey of the time kinetics of TNF killing at various temperatures utilizing the more sensitive modification of the basic microassay is shown in Fig. 22. Despite their greatly enhanced susceptibility to TNF when cultured in the presence of actinomycin D, L-929 cells are still refractory to the lytic effects of TNF at 25°. As the temperature is elevated the onset of cell killing is accelerated, as well as the rate at which the cells die. Cells grown at 39° showed substantially more lethality than their 37° counterparts at the end of 10 hrs. The temperature dependence of killing is consistent with the idea that metabolically active cells are better targets for TNF action. The temperature dependence is also consistent with the enzymatic mode of action suggested by the slope of the dose curves.

#### Effects on Macromolecular Synthesis

In a simple sense we knew that ultimately TNF must be inhibitory to all anabolic pathways. We did not know if cell death occurred directly as a result of TNF mediated shut down of host synthesis or whether inhibition was the repercussion of an earlier, yet to be identified, lesion. If the ability of cells to synthesize DNA or protein is measured by incorporation of labeled precursors in the continuous presence of TNF, shutdown of these processes is delayed by approximately 12 hours and proceeds at a rate comparable to loss of cell recovery. Ostrove and Gifford (1979) described a very interesting paradoxical increase in  $^3\text{H}$ -uridine incorporation in TNF treated L929 cells. A sixfold stimulation of RNA synthesis was reported 24 hours after TNF addition. Normal mouse embryo fibroblasts, or clones of L cells selected for their relative resistance to TNF, did not show this effect. They further reported the synergistic effect of actinomycin D

Figure 22. The time kinetics of L-929 cell killing at different temperatures was determined in the presence of actinomycin D.



on TNF induced cell killing which was interpreted to imply a repair capability. New transcription may be required to repair or replace a TNF damaged cellular component. We have shown that  $\alpha$ -amanitin, an inhibitor of RNA polymerase II, will also enhance TNF induced cell killing, further strengthening the role for a newly synthesized mRNA as a component of the proposed repair process.

Although cells may respond to TNF by the prescribed repair route, it is difficult to explain a six-fold increase in  $^3\text{H}$ -uridine incorporation as being due to amplification of a particular mRNA species. Furthermore, the burst of synthesis occurs rather late in the course of TNF action, 24 hours, since in the absence of just such a burst of repair activity (actinomycin D treated) cells are already dying by four to six hours. Any reparative mechanism, to be physiologically relevant, must be initiated within the first few hours after TNF addition. Enhanced RNA synthesis may indeed be a component of TNF action but at present time no definite interpretation is possible. In this regard, it is interesting to note that lymphotoxin also stimulates RNA synthesis in target cells and the cytolytic events are also accelerated by treatment with actinomycin D (Rosenau et al., 1973; Kunitomi et al., 1975).

#### Cell Cycle or Growth Dependent Sites of Action

Some of the early experiments (Ruff and Gifford, 1980a) were designed to more closely examine the specificity of TNF action for transformed vs. normal cells. We wondered if normal cells, by virtue of their density dependent growth properties, might be refractory to TNF action because they were relatively quiescent as a result of the high initial seeding densities in cultures. Cells were thus plated in

the presence of TNF so that multiple rounds of division would be required to reach confluency. The results, Table V, show that TNF inhibited L- 929 cells by 80% at all seeding densities tried. Mouse embryo fibroblasts were resistant to TNF whether they went through two ( $12.5 \times 10^3$  cells/culture), one ( $25 \times 10^3$  cells/culture), or less than one ( $50 \times 10^3$  cells/culture) division. The stimulatory effect of TNF serum on normal cells is more pronounced on metabolically active, dividing cultures. This effect is probably due to growth promoting agents present in the crude serum unrelated to TNF action.

Since cell growth and division were not discriminatory factors in the specific action of TNF we wished to consider the effect of cell growth rate on sensitivity to TNF. L-929 cells seeded in the presence of TNF in various concentrations of serum showed a graded response of cell killing (Fig. 23). Cells grown in low serum, 2.5%, are more resistant to cytolysis than cells grown in higher serum. Doses of serum greater than 10% do not enhance cell killing. The serum dependency of TNF action can be overcome by adding increasing amounts of TNF. Matthews and Watkins (1978) have reported that cells blocked from dividing by the DNA cross-linking agent mitomycin C were still killed by TNF. They further indicate that if cells were treated with the electron transport inhibitors azide or dinitrophenol, the effectiveness of TNF action was reduced. The inhibition of killing could be overcome by higher doses of TNF. Our initial impression is that TNF may have as its target of action a division related metabolic pathway. Cells forced to slow down their progression through the cell cycle by serum deprivation or metabolic inhibitors become less sensitive to TNF. Clearly cell killing can proceed in the absence of any cell division or even in the absence of RNA transcription or protein synthesis. We have



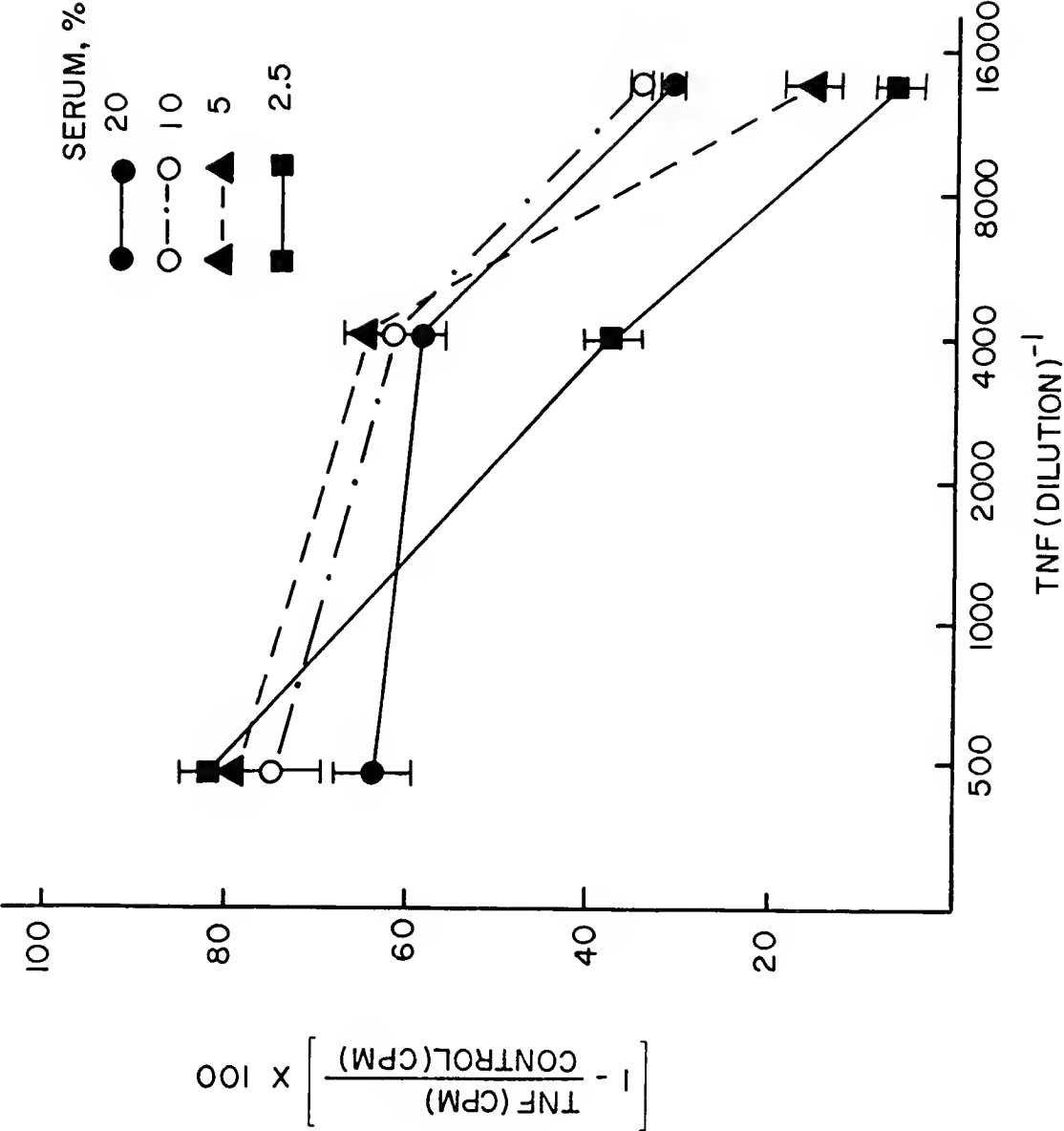
TABLE V

The Effect of Plating Density on TNF Cell Killing of  
Normal and Transformed Cells

Cell Type	Plating Density/Culture (cells x 10 <sup>-3</sup> )		
	50	25	12
MEF	1.17*	1.33	1.82
L-929	.21	.20	.21

\*Numbers represent the ratio of <sup>3</sup>H-thymidine incorporation in TNF/Control cultures at the end of 48 hours. Cultures were treated with TNF serum at a dilution of 1:200 at the indicated plating densities.

Figure 23. The serum dependency of TNF cell killing was determined by culturing cells in the presence of different amounts of fetal calf serum. Survival ratios were made at the end of a 48 hour culture period.



proposed (Ruff and Gifford, 1980b) that cells may vary in their sensitivity to TNF during their growth cycle. Specifically a time shortly after mitosis was suggested as a period when cells might show enhanced TNF killing effects due to demand for new membrane components which accompanies segregation of daughter cells. This was suggested by time-lapse movies of TNF treated cells by Saul Green (personal communication) and ourselves which showed many, but not all, cells dying shortly after mitosis. Other interpretations of these observations are possible. Cikes and Klein (1972) have described the re-expression of surface antigens following re-feeding of stationary phase YAC lymphoma cells. Metabolically inhibited cells may simply have diminished numbers of TNF surface receptors.

#### Possible Binding Interactions

It seems reasonable to conjecture that binding of TNF to target cells was a likely requisite of its action although precedent for alternative mechanisms had been established. The soluble macrophage cytotoxin described by Currie and Basham (1975) was later shown to have arginase activity (Currie, 1978). Many years earlier Broome (1961) reported that the anti-lymphoma properties of guinea pig serum were due to high levels of L-asparaginase. We have found that supplementation of the culture medium with 5x amino acids does not inhibit TNF action. Further, media which was dialysed against TNF serum for several days did not show the growth inhibiting activity of TNF, nor did it result in the depletion of 18 media containing amino acids when they were assayed for directly.

#### Neutralization by Sugars and Lectins

Neutralization of cytotoxin activity by sugars or glycopeptides has often proved useful in revealing their site of action.

Thus Pincus et al. (1971) have shown that N-acetylgalactosamine and N-acetylglucosamine neutralize a macrophage cytotoxin but have no effect on lymphotoxin. Similarly, Kobayashi et al. (1978) have recently applied this approach to define the putative lymphotoxin glycoprotein surface receptor. Matthews and Watkins (1978) and ourselves have tried to block rabbit TNF action on L cells with several sugars and plant lectins to gain an insight on the nature of the substrate or receptor. None of the substances tested had any inhibiting effect on TNF activity (Table VI).

#### Inhibition by Membrane Proteins

A more direct approach at defining the TNF receptor was tried. Crude plasma membrane components were isolated by the two-phase aqueous polymer system of Brunette and Till (1971). This procedure provides a high yield 10-fold enrichment of membrane over cytoplasmic and nuclear proteins. Membrane proteins solubilized by treatment with the detergent NP40, 1%, for three hours, were dialysed and assayed for their ability to presumably compete with endogenous cell receptor and thereby block TNF killing. We were able to inhibit cell killing by 70% with this method; however the membrane protein equivalent of  $10^6$  L-929 cells was required to attain this level of protection in the standard assay. Although this approach may have promise our feeling is that we have not defined conditions which allow isolation of native membrane components with intact receptor activity. It is entirely possible that, as in the case with the epidermal growth factor-urogastrone receptor (Hock et al., 1979), the proposed TNF receptor may lose its ligand recognizing ability after solubilization with detergents.

TABLE VI<sup>a</sup>

Sugars and Lectins Tested for Their Effect on TNF Activity

Sugars	Lectins <sup>d</sup>	Sugar Specificity
$\alpha$ -methyl mannose <sup>b,c</sup>	Arachis hypogaea (peanut)	D-gal- $\beta$ (1 $\rightarrow$ 3) galNac
N-acetyl glucosamine <sup>b</sup>	Concanavalin A	$\alpha$ -D-glc, $\alpha$ -D-man
D-galactose <sup>b,c</sup>	Lens culinaris (lenti)	$\alpha$ -D-glc, $\alpha$ -D-man
fucose <sup>b,c</sup>	Lotus tetragonolobus	$\alpha$ -L-fucose
$\alpha$ -D-mannose <sup>b,c</sup>	Phaseolus vulgaris (PHA)	D-galNac, others (?)
$\alpha$ -D-galactose-lactone <sup>b</sup>	Ricinus communis I,II	$\beta$ -D-gal, D-galNac
N-acetyl galactosamine <sup>b,c</sup>	Triticum vulgaris (wheat germ)	sialic acid
D-glucosamine <sup>b</sup>		
lactose <sup>c</sup>		
N-acetyl neuraminic acid <sup>b</sup>		

<sup>a</sup>Lectins and sugars were tested in culture, at the indicated concentrations, for their ability to inhibit TNF cell killing.

<sup>b</sup>1 mg/ml (Ruff and Gifford, 1980a)      <sup>c</sup>50  $\mu$ g/ml (Matthews and Watkins, 1978)

<sup>d</sup>10  $\mu$ g/ml (Ruff and Gifford, 1980a)

## Cytoskeletal Aspects of TNF Action

### Internationalization of TNF

Macromolecules are taken into the cell by the process of receptor mediated endocytosis (reviewed by Goldstein et al., 1979). In this model specific receptors, located largely within distinct membrane structures known as "coated pits," serve as internalization sites by the process of endocytosis. The endocytic vesicle is delivered to the lysosome where the endocytosed proteins are usually completely degraded to amino acids. The two major cytoskeletal systems, the actin containing microfilaments, and tubulin containing microtubules are generally envisioned as playing important roles in the process of internalization and delivery.

Cytochalasin B inhibits cell movement, cytoplasmic streaming, and membrane movements, defects considered to be due to impairment of structural microfilaments. Capping of surface receptors, an event which usually precedes internalization, is also inhibitable, although to variable degrees in different systems, by cytochalasin B, (reviewed by de Petris, 1977). Phagocytosis by macrophages has also been shown to be inhibitable by cytochalasin B. (Axline and Reaven, 1974). In at least one system, that for internalization of low density lipoproteins, coated pits have been shown to overlay the submembranar actin web (Anderson, et al., 1978). Microfilaments are thus presumed to mediate the initial stages of phagocytic vacuole formation and internalization; however, the mechanistic details of these processes have yet to be resolved.

Microtubules have also been reported to play an integral role in endocytic process, as well as secretion (reviewed by Dustin, 1978) and have been implicated in the action of some lymphokines, such as MAF

(Pick and Abrahamer, 1973). Colchicine, which binds to tubulin causing its depolymerization, has been found to interfere with polymorphonuclear chemotaxis, formation of phagosomes, and release of enzymes into these vacuoles (Malawista, 1975). Colchicine also suppresses insulin release from pancreatic B cells (Lacy et al., 1968, Lacy and Melaisse, 1973), thyroid hormones from TSH treated thyroid cells (Neve et al., 1970), and has similar effects in other secretory systems. Microtubules are envisioned as contractile elements involved in the movement of endocytic vesicles and lysosomes which ultimately result in formation of the phagolysosome, or in the movement of the secretory granule to the cell surface where it is exocytosed.

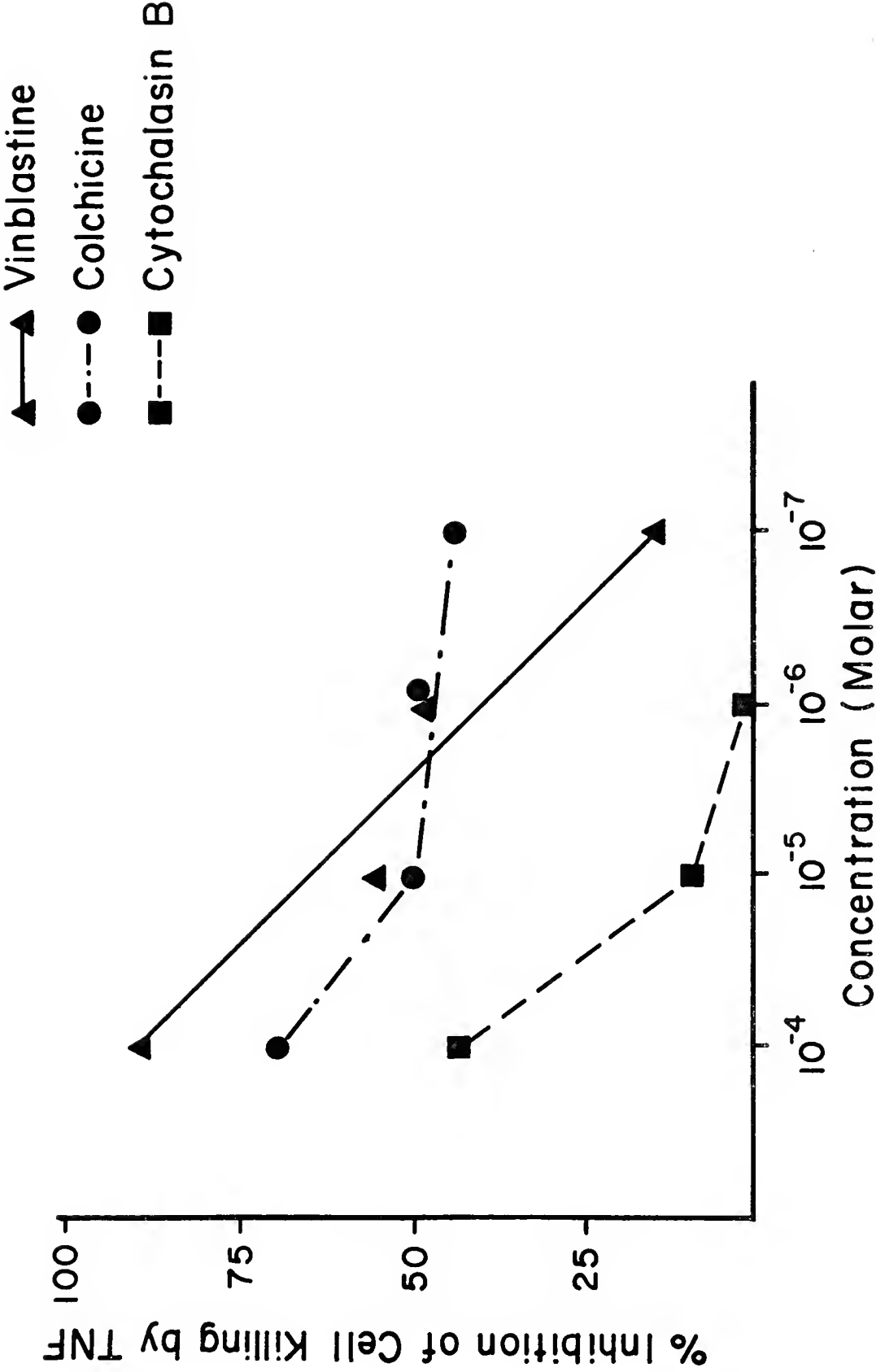
Cells treated with TNF in the presence of the microfilament antagonist cytochalasin B, or the microfilament antagonists colchicine or vinblastine, showed dose dependent decreases in cell killing (Fig. 24). On a molar basis the microtubule inhibitors were more effective at protecting than the microfilament inhibitor, although this may merely reflect permeability differences among the various agents. These experiments argue that TNF most likely gains entrance to the internal cellular milieu by the process of receptor mediated endocytosis and furthermore it must do so to exert its cytolytic effects.

#### TNF: A Possible Zymogen or Cytotoxinogen

Since the usual fate of an endocytosed protein is delivery to lysosomes where it is completely degraded (Carpenter and Cohen, 1976; Wibo and Poole, 1974; Goldstein et al., 1979), we questioned what contribution lysosomal function might have on TNF mediated cell killing. Chloroquine is a lysomotropic agent (DeDuve et al., 1974)



Figure 24. Inhibitors of microtubules, vinblastine and colchicine, and an inhibitor of microfilament assembly, cytochalasin B, were evaluated for their effect on TNF cell killing. Inhibitors were added simultaneously with TNF at the beginning of culture, in the presence of actinomycin D. Destruction of the L-929 target monolayers was evaluated in 18 hours.



which has been shown to inhibit lysosomal enzymes functioning (Lie and Schofield, 1973; Wibo and Poole, 1974). Degradation of endocytosed protein in a wide variety of systems has been inhibited by this agent (Carpenter and Cohen, 1976; Ascoli and Puett, 1978; Goldstein et al., 1979).

Cells treated with purified TNF, in the presence of actinomycin D and chloroquine,  $10^{-4}M$ , showed a 95% inhibition of cell killing over controls. These results imply that lysosomal enzyme functioning is necessary for TNF action. Perhaps TNF might act as a labilizer of lysosomal membranes causing their release within the cell. Such an event would have significant impact on normal cellular physiology and would be consistent with the proposed "anabolic" mode of TNF action (see "Effects on Macromolecular Synthesis"). Serum TNF might exist as a zymogen which, once having entered the cell, is activated by lysosomal enzymes. A speculative argument for how "activated TNF" might kill cells is suggested by work done by Sintex and Pincus (1970). Thioglycollate induced peritoneal cells from tuberculin positive guinea pigs could be induced with PPD to secrete a cytotoxic factor for L cells. Cytotoxic activity was shown to be due to a phospholipid which apparently could associate with a 30-40,000 d protein. The cytotoxic activity could be dissociated from the protein carrier at pH 5.0. Thus, could the protein component of TNF serve as a specific carrier for such a phospholipid toxin, targeting it to specific cells and providing the vehicle for internalization? Once within the cell lysosomal hydrolases would release the toxic component from the carrier. This argument does not, however, address the proposed enzymatic mode of TNF action. Alternatively could TNF exist as a

zymogen in the more traditional sense, that of pro-cytoxin, perhaps pro-enzyme, independent of any cryptic, nonprotein toxin?

#### TNF - A Metalloprotein

A model for cell killing whose general features incorporated both the specificity and catalytic properties of an enzymatic reaction was intriguing from the start. The less than first-order kinetics of the dose response curve was the first compelling reason to suggest such a mechanism. The temperature sensitivity of TNF action is superficially consistent with such a model. In an attempt to further strengthen this argument we screened several inhibitors of specific protease classes for their effect on TNF action. These results are listed in Table VII.

Soybean trypsin inhibitor is a protein macromolecule which forms a poorly dissociating complex with trypsin-like proteases in molar amounts (for specific inhibitors see Boyer, 1971). N-tosyl-L-lysine chloromethyl ketone (TLCK) is a substrate analog which reacts covalently and irreversibly with trypsin-like enzymes. A more general non-competitive inhibitor of the serine protease family, of which trypsin and plasminogen activator are members, is phenylmethyl sulfonyl-flouride (PMSF). None of these compounds inhibited TNF activity, nor did  $\epsilon$ -amino caproic acid, an inhibitor of plasmin. These compounds were tested against purified TNF at concentrations up to the cell toxicity limits. The chealting agent o-phenanthroline, at concentrations of  $10^{-5}$  in culture, was however able to block TNF cell killing by greater than 99% (Fig. 25). The inhibitor is specific for metal containing endo- and exopeptidases and by virtue of its high affinity for transition metals will compete for the metal ligand, thereby inactivating the enzyme (Vallee and Wacker, 1970; Boyer, 1971).

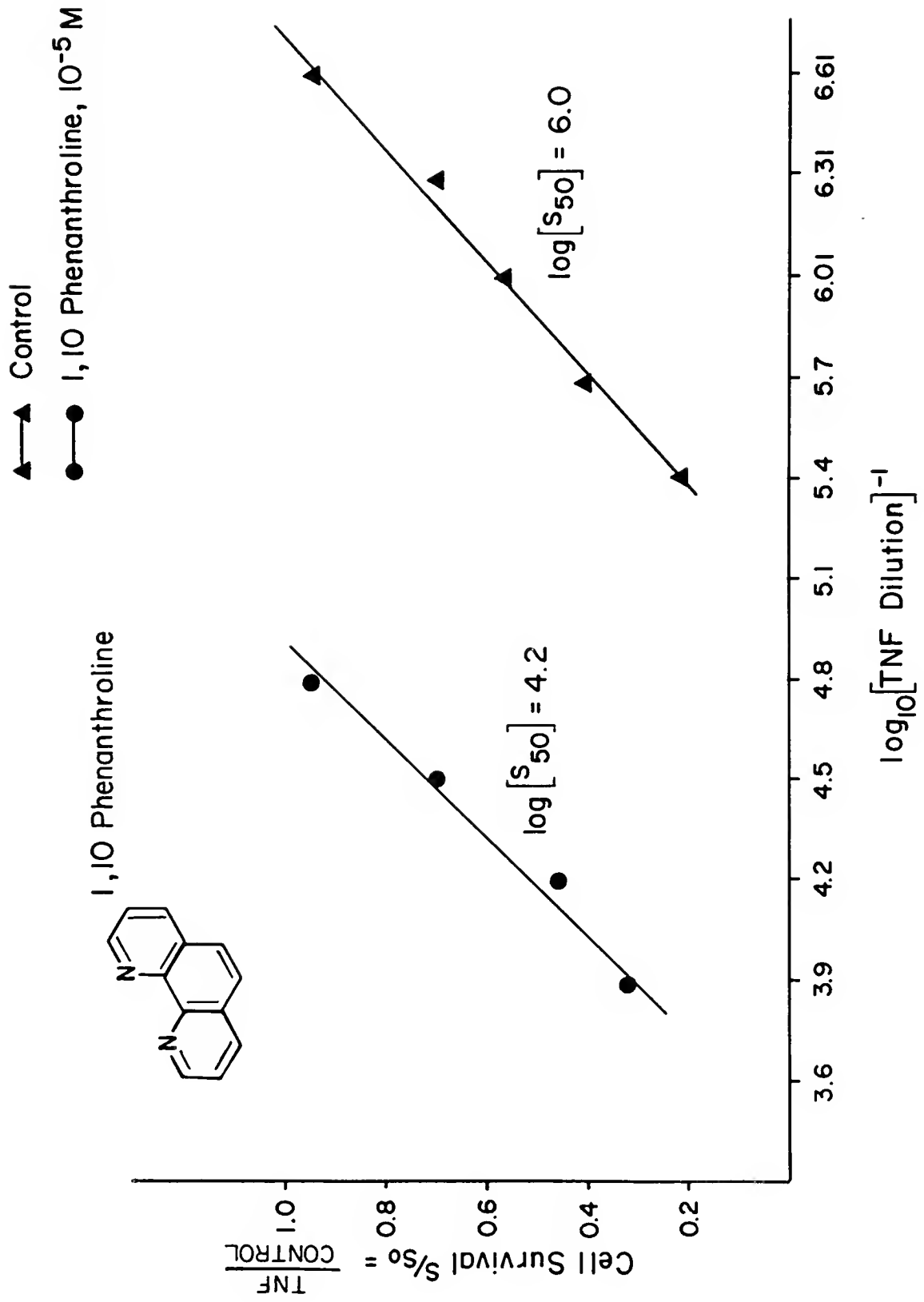
TABLE VII<sup>a</sup>

Protease Inhibitors Tested for Their Effect on TNF Cell Killing

	Concentration	Specificity
Soybean Trypsin Inhibitor	100 µg/ml	Trypsin-like
N-Tosyl-L-Lys Chloromethyl Ketone (TLCK)	10 <sup>-4</sup> M	Trypsin-like
Phenylmethyl-sulfonylflouride (PMSF)	10 <sup>-4</sup>	Serine Protease
ε-aminocaproic Acid	60 µg/ml	Plasmin
o-phenanthroline	10 <sup>-5</sup>	Metal Containing

<sup>a</sup>The above listed compounds were tested for their effect on TNF mediated cell killing in the 18 hour killing assay as detailed in Methods. Final concentrations in culture are listed.

Figure 25. The metal chelator, o-phenanthroline, was added to cultures containing various dilutions of TNF. The effect on the dose kinetics of killing was determined at the end of 18 hours in the presence of actinomycin D.



The inhibition by o-phenanthroline is 100% reversible with molybdenum and copper salts, and 40% restorable with cobalt salts. Other metals tested did not restore cell killing by TNF in the presence of the inhibitor (Table VIII). Sodium azide is a strong chelator for transition and IIB elements, particularly iron, copper, and zinc (Vallee and Wacker, 1970). We have already discussed the inhibition of TNF action by azide (Matthews and Watkins, 1978) and suggest that this is due to its chelating abilities. If TNF does contain a metal binding site, copper would seem to be a likely candidate. Since these trace elements represent variable and undefined components of the culture media, which presumably are required for cell vitality, we have been hampered in our attempts to perform the dialysis exchange type of experiments which might conclusively implicate a specific metal component of TNF (as opposed to a co-factor, or cellular, heavy metal dependency). Other chelators such as EDTA, iminodiacetic acid and imidazole did not inhibit TNF activity in culture but this may be due to their appreciable binding with the group IIA and IA elements, many of which are present in high molar amounts in tissue medium.



TABLE VIII  
Effect of Heavy Metal Ions on TNF Cell Killing

Culture Additions <sup>a</sup>	Metal Additions <sup>b</sup>								
	None	Fe	Co	Mn	Mg	Cu	Cr	Zn	Mo
None	100	99	102	100	100	98	102	99	103
o-phenanthroline	106	105	103	96	96	47	99	98	100
TNF	10	6	8	17	6	7	7	13	7
TNF + o-phenanthroline	42	43	18	40	53	8	41	42	10

<sup>a</sup>Cells were cultured in the presence of o-phenanthroline,  $10^{-5}$  M and various metal salts at  $10^{-4}$  M, with or without TNF, as indicated.

<sup>b</sup>Numbers represent survival ratios for experimental/control incubations expressed as a percent of control.

## DISCUSSION

Rabbit TNF has been purified 2000 fold (Table II) to a largely single homogeneous species on SDS-PAGE with a molecular weight of 68,000 daltons (Fig. 6). The size estimate by this determination is approximately 25% larger than the values determined under non-denaturing conditions by gel filtration (Fig. 5) or glycerol gradient centrifugation (Fig. 8), where estimates of 52,000 and 55,000 were obtained. Although the size estimates by the respective techniques are reproducible, the magnitude of the observed variations makes an unequivocal decision regarding their significance difficult and this variation may merely reflect the inherent limitations of the methodologies employed. In this context it is tempting to speculate whether SDS denaturation might reveal an alternative configuration for TNF which has some significance in terms of its biological activity. Alternatively the protein may have less hydrophobic character than most proteins and would thus bind less SDS, resulting in an apparent shift toward higher molecular weight when this determination is made via SDS-PAGE. Such a phenomenon has been observed for the A fragment of diphtheria toxin (Boquet et al., 1976) and a similar argument was proposed to explain size differences between human and nurse shark J chains (McCumber and Clem, 1976). In non-denaturing PAGE, TNF activity was recovered from the gel in a single band to the anodic side of the BSA marker, in the  $\alpha$ -globulin region of the gel (Fig. 7). In non-denaturing PAGE, TNF activity was recovered from the gel in a

single band to the anodic side of the BSA marker, in the  $\alpha$ -globulin region of the gel (Fig. 7). In this respect rabbit TNF is similar to mouse TNF (Green et al., 1976).

Partially purified mouse TNF has been reported to be a sialic acid containing glycoprotein (Green et al., 1976). Rabbit TNF was not bound to affinity columns prepared from Ricinus comunis lectin or PHA, which would be expected to have the appropriate binding specificities for sialic acids (Ruff and Gifford, 1980a). Additionally, neuraminidase treatment did not destroy TNF activity (Table III). These data suggest that if rabbit TNF is a glycoprotein it may not contain sialic acid and presumably does not contain mannopyranosyl or related residues, as judged by its failure to bind to Con A affinity columns.

The sensitivity of TNF to hydrolysis by several other enzymes was evaluated in order to better understand the physical composition of the molecule. The complete loss of activity after pronase digestion confirms the protein nature of the molecule. The failure to destroy TNF activity by trypsin, except after prolonged digestion, would suggest that TNF may have few exposed basic amino acids. This would be consistent with the observed low affinity of TNF for cation exchange resins such as CM or SP Sepharose. Sintex and Pincus (1970) reported the isolation of a cytotoxin from peritoneal cells which was composed of a phospholipid in association with a protein carrier. The activity of their preparation could be destroyed by incubation with phospholipase C. TNF is presumably different from this toxin since activity was stable to phospholipase C digestion.

Rabbit TNF maintained activity after heating at 70° although mouse TNF is unstable under these conditions (Green et al., 1976). The protein is also relatively resistant to high pH although it

rapidly loses activity at slightly acid pH. In working with the protein we have also ascertained that it will survive storage at 4° for three months with minimal loss of activity. Some of the activity loss during purification may be attributable to lysozomal hydrolases known to be present in high amounts in TNF serum (Green et. al., 1976). TNF activity was completely destroyed by treatment with the reducing agent  $\beta$ -mercaptoethanol,  $10^{-5}$  M, or precipitation with trichloroacetic acid or acetone.

Crude rabbit serum TNF as well as a partially purified preparation were capable of eliciting a hemorrhagic reaction within the Meth A tumors of susceptible mice. In a limited series of trials 1<sup>+</sup> and 2<sup>+</sup> necrotic reactions were observed but no tumors completely regressed. Whether purified TNF will still elicit the hemorrhagic necrosis of susceptible tumors has yet to be determined.

The slowly progressing cytostatic and cytocidal effects of TNF are similar to those described for lymphotoxin, a cytotoxic protein which seems to be elaborated by T lymphocytes in vitro following mitogenic stimulation (Rosenau et al., 1973; Kunitomi et al., 1975). A major difference however is that lymphotoxin is not discriminatory for transformed cells but is lytic for both normal and neoplastic cells (Granger, 1969; Williams and Granger, 1969). Lymphotoxin also lyses red blood cells (Granger, 1969) but we find no lysis of these cells by TNF. Additional similarities exist between lymphotoxin and TNF action. Both lack species specificity and both result in a paradoxical increase in RNA synthesis in treated cells (Ostrove and Gifford, 1979; Rosenau et al., 1973; Kunitomi et al., 1975). Lymphotoxin has also been reported to have a serum requirement for action (Lies, 1974).

Currie and Basham (1975) have shown that a soluble supernatant fraction can be isolated from endotoxin activated peritoneal macrophages which kills malignant but not normal cell (1978). Currie has subsequently shown that this factor has arginase activity. These data are reminiscent of earlier work describing an anti-lymphoma agent present in the serum of normal guinea pigs, later shown to be an asparaginase (Broome, 1961). We had considered whether TNF might exert its selective killing effects by a mechanism similar to the soluble enzymes just described. Media which had been dialysed against TNF serum for four days did not however show any anti-L929 cell activity nor was there any decrease in 18 media containing amino acids when they were assayed for directly. Supplementation of media with additional vitamins and amino acids did not inhibit TNF action (data not shown). We therefore feel that TNF killing is not the result of nutritional depletion but rather it must interact directly with the sensitive cells.

Transformed cells differ from normal cells in many respects, some of the most pronounced being the loss of growth controls. We questioned whether these phenotypic differences might serve as the basis for the selectivity of TNF action. Was TNF less active on growth arrested cells? Normal cells plated in the presence of TNF containing serum and allowed to go through multiple rounds of division were still resistant to TNF action (Table I). Transformed cells, typically unrestrained in their growth characteristics, could be made to slow down their transgression through the cell cycle by serum deprivation or actually blocked through agents such as mitomycin C or low temperature. Division blockage with mitomycin C did not inhibit TNF activity on L929 cells, an observation also reported by Matthews (1978). Transformed

cells grown in the presence of low serum do show a decrease in TNF sensitivity (Fig. 5) although this inhibition is only partial and can be overcome with increased amounts of TNF. Since cell killing effectively proceeds in the absence of major anabolic pathway activity, and in fact shows synergistic activity in the presence of the inhibitors actinomycin D and cycloheximide (Fig. 1), we feel that a cell cycle dependent mechanism of action is unlikely for TNF. Moreover the inhibitor data would argue that TNF exerts its effects by actively degrading or inactivating an essential cell component, perhaps structural. The cycloheximide sensitivity would suggest a protein target.

The serum dependency of TNF action might however suggest that sensitive cells vary in their susceptibility to TNF as a function of their position in the growth cycle. For example, time lapse cinematography of TNF treated cultures (in the absence of any inhibitors) shows many, but not all cells proceeding through mitosis after which one or both daughter cells become highly granular, round up and detach from the surface (S. Green, personal communication and data not shown). It seems reasonable to conjecture that TNF could act at the membrane level since membrane leakiness is an early component of TNF action (Fig. 14). Thus, shortly after mitosis when the cell undergoes an increased demand for membrane components as new membranes are synthesized and assembled, and the daughter cells expand to their mature cell volume, they might be expected to show an enhanced sensitivity to TNF. Serum starvation protects by slowing the rate at which cells enter this "crisis" period. An alternative explanation for the serum dependence of TNF action is suggested by work done by Cikes and Klein (1972)

who have described the re-expression of surface antigens following re-feeding of stationary phase YAC lymphoma cells. Serum starved cells may simply have diminished numbers of TNF binding sites. As was suggested for lymphocytes action (Williams and Granger, 1969), a serum component might also be directly required for TNF activity.

The temperature dependence of TNF killing could also be discussed within the framework of the above arguments, since low temperatures would be expected to inhibit cell growth and metabolism. Relevant metabolic processes involved in TNF action could perhaps be uptake or endocytosis of cell bound TNF, activation or further cell mediated processing of TNF, since an internal site for TNF action cannot be excluded by the available data.

While formulating models of toxin action we were most interested in obtaining insights into two general areas, the nature of TNF discriminating cell killing and the mechanisms by which the cell killing proceeded. An analysis of the types of perturbations present in TNF treated cells could provide information on possible targets or sites for expression of TNF activity, which would perhaps resolve the more basic questions. It has been argued above, and in the body of the text, that TNF most likely destroys or inactivates a vital cellular component and that cells are not killed merely due to inhibition of requisite anabolic processes, at least on a gross level. Various parameters of TNF action, such as possible division or cell cycle related targets , as well as the serum and temperature dependencies, have been discussed within the context of particular sites or modes of cytotoxin activity.

immediately. In practice neither of these predictions were substantiated (Fig. 20). The rate of cell killing was independent of the amount of TNF which was added to the cells over the almost 200-fold range which we considered. Additionally, a dose of TNF which was  $10^4$  times greater than the amount needed to kill 50% of the cells in 18 hours did not overcome the four hour lag which precedes the onset of cell death.

The interpretation of these findings was that TNF had to gain entrance to the intracellular compartment to express its killing effect. The lag represents a minimum time required for the cell to achieve a steady state concentration of TNF incompatible with continued viability and we further argued that since the rate of cell killing seems to be independent of TNF concentration serum TNF might not be the actual cytotoxic mediator. TNF mediated release of intracellular hydrolytic enzymes was suggested as one possibility consistent with the available information. An equally plausible alternative would be that the high amounts of TNF which were employed by way of testing for the inviolability of the lag period, having once established an intracellular steady state, were still in great excess of the amount required to kill a cell. A corollary of this argument would be that much higher amounts of TNF are needed to saturate the proposed cellular uptake system compared to the amounts needed to exert a biological effect, namely cell destruction. Hormone action in many systems follows this paradigm.

In the absence of highly purified TNF, which could be isotopically tagged and used to follow binding and internalization events, a number of indirect methods were employed to decide whether the protein must necessarily be internalized as a requisite to its action. Several



inhibitors of receptor mediated transport processes were examined for their effect on TNF action. Both microfilament and microtubule antagonists, cytochalasin B, or vinblastine and colchicine, protected cells to TNF cytotoxicity. The mechanistic details of endocytic processes are poorly understood at present and consequently the interpretation of these inhibitor results are not unequivocal. Microfilaments probably mediate initial stages of endocytic vacuole formation. Within this context TNF could still be considered to be "outside" the cell. Microtubule inhibitors block fusion of the endocytic vesicle with lysosomes (Brown et al., 1980), although not all macromolecules follow this pathway of internalization (Ostlund et al., 1979; Middlebrook, et al., 1979). In this sense TNF would be "inside" the cell, although still effectively partitioned from the cytosol, and presumably its target of action.

Since endocytosed protein is usually delivered to the lysosome where it is completely degraded (Goldstein et al., 1979) we evaluated lysosomal functioning in relation to TNF activity, once again, by an indirect method. Chloroquine, a lysomotropic agent, completely protected the cells to TNF killing. This observation has several interpretations. Lysosomal enzyme functioning could be important for TNF action, either to directly activate or process "pro-TNF" (Zymogen theory) as the actual agent of cell dissolution by a scheme which envisages a TNF mediated intracellular degranulation of lysosomes. Alternatively, lysosomes which have sequestered large amounts of chloroquine may not be able to complete the process of fusion with endocytic vesicles. TNF would remain compartmentalized within the cell, similar to the situation which occurs with colchicine. Finally, at this time it is not possible to exclude a direct effect of

TABLE IX<sup>a</sup>

Synthetic Protein Analogs Tested for Hydrolysis by TNF

Analogs
$\alpha$ -N-p-Tosyl-L-Arginine Amide L-1-Tosylamide-2-Phenylethyl-chloromethyl Ketone (TPCK) p-Tosyl-L-Arginine Methyl Ester (TAME) N-Tosyl-L-Glutamic Acid p-Tosyl Glycine p-Tosylglycine Benzyl Ester p-Tosylglycylglycine Benzyl Ester p-Tosyl-L-Leucine Benzyl Ester N- $\alpha$ -p-Tosyl-L-Lysine Chloromethyl Ketone (TLCK) N- $\alpha$ -p-Tosyl-L-Lysine Methyl Ester p-Tosyl-L-Phenylalanine Benzyl Ester p-Tosyl-L-Tyrosine Benzyl Ester  Hippuryl-L-Arginine Hippuryl-L-Histidyl-L-Leucine Hippuryl-L-Lysine Hippuryl-L-Phenylalanine

<sup>a</sup>Hydrolysis was monitored spectrophotometrically for 2 hours by incubating analogs at a concentration of 1mM with purified TNF at a final concentration of 10  $\mu$ g/ml at 37<sup>o</sup>. Tosyl analogs were monitored at 247 nm, Hippuryl analogs at 254 nm. No significant hydrolysis was observed for any of these compounds.

chloroquine on TNF itself since chloroquine has been reported to directly inhibit some members of the cathepsin protease family (Lie and Schofield, 1973; Wibo and Poole, 1974).

We felt that TNF might have an enzymatic mode of action and were interested in pursuing this avenue of approach. Concentrations of TNF on the order of  $10^{-9}$  M were capable of effectively killing cells in 18-24 hours. Over a period of several days amounts of TNF orders of magnitude lower would be expected to exert substantial killing effects. The low slopes of the dose curves, as discussed, would support such a notion, as would the temperature sensitivity of cell killing. Initially, we felt that TNF might itself be a protease, partly because macrophages have been reported to secrete several proteases, some of which seemed to be involved in lysing tumor targets but not normal fibroblasts (Adams, 1980) and partly because cell killing by TNF was accelerated and enhanced in the presence of cycloheximide, a protein synthesis inhibitor. All of the macrophage cytotoxins which have been described as having protease activity have been of the serine esterase type. None of the inhibitors of this class of protease had an effect on TNF mediated cell destruction. We further screened a variety of artificial protease substrates for possible hydrolysis by TNF (Table IX) but results were negative.

The metal chelator o-phenanthroline did inhibit TNF activity and we went on to show that the protein most likely contains a copper binding ligand. Since all of the enzymes of electron transport and  $O_2$  scavenging contain metal binding ligands, many for copper, iron, and zinc, we questioned if TNF might also be involved in electron trafficking as a free radical generator. Specifically we wondered if TNF might be a generator of superoxide anion,  $O_2^-$ . If this were

the case scavengers of superoxide or the spontaneous dismutation product,  $H_2O_2$ , should be capable of protecting cells against TNF killing. This turned out to be the case, as catalase and peroxidase were capable of completely protecting the cells to TNF killing.

The studies on the possible enzymatic activity of TNF were instituted at the time of this writing and although they are quite incomplete were discussed now to provide (perhaps) a modicum of completeness. Some notion of TNF mechanism of action would be very satisfying at this juncture. Many elements of TNF interaction with cells, its possible binding, internalization and metabolism have been described only inferentially. The two major questions which were asked, how does TNF discriminate in its killing action and how might killing be effected are largely unresolved. What has been accomplished is a fairly complete description of how the cell responds to TNF, including the kinetic time frame of such activity. Several theories based on other toxin models of how TNF might work have been discarded. The protein has been purified and something is now known of its physical characteristics. The fact that TNF may be a metalloprotein has strongly focused on direction of inquiry on how it may operate, and it now seems likely that the mechanistic details may be revealed in the course of my lifetime, although perhaps not by me. The availability of a radiolabeled, purified protein will put questions of possible TNF binding, internalization and metabolism on more secure foundation. These further studies, interpreted within the framework of the results discussed in this dissertation, now provide an intelligible and sensible scheme by which to approach the remaining questions concerning TNF action, particularly the basis of discriminatory cell killing.

## PROSPECTUS

Activated macrophages generally show non-specific anti-microbial or tumoricidal capacity. It is now appreciated that the conversion of the mononuclear phagocyte from the "resting" state to a state of "activation" occurs as a result of appropriately timed activation signals. Activation is not a single step but rather represents a concerted process of differentiation, each stage subject to various regulatory controls. Several intermediate stages preceding ultimate activation have been defined in in vitro model system. Activating signals can be both non-immune, such as BCG, endotoxin, and other adjuvants, and immune, such as specific lymphocyte products, underscoring this dichotomy of RES effector function.

A cytotoxic factor(s) which shows discriminating toxicity for transformed cells over normal cells can be elicited from macrophages by techniques which have traditionally been used to generate broadly cytotoxic macrophages. Activation of cells for TNF production seems to follow the general paradigm developed for activation of macrophages to tumoricidal capability. Multiple stages in TNF production have been identified, and production can be controlled by both immune and non-immune signals.

RES adjuvants, such as BCG, provide an initial activating signal. Macrophages are known to undergo various biochemical alterations which render them tumoricidal as a result of this treatment. Additionally experimental tuberculosis, and other adjuvant treatments in animals,

results in a dramatic expansion of monocytic cells. RES expansion may be achieved by induction of a particular lymphokine, bone marrow colony stimulating factor (CSF). CSF induces macrophage maturation and expansion in the liver and spleen, and other tissue sites, including tumor masses. Other lymphokines, such as migration inhibiting factor (MIF) may serve to localize macrophages to sites of tissue injury.

Final activation of BCG "primed" macrophages can be achieved with endotoxin, or priming can be bypassed with a lymphokine, perhaps macrophage activating factor (MAF), present in mitogen stimulated lymphocyte supernates. Under these conditions macrophages become maximally tumoricidal and also release a variety of biological effector molecules, such as interferon, lymphocyte activating factor (LAF), and TNF. These soluble agents have been shown to have both inhibitory and stimulatory action on specific T-cell killing activities. These or other monokines also have direct effects on neoplastic cells themselves. Ultimate macrophage activation, including overt tumoricidal capability, as well as TNF production, is an evanescent state, lasting for only several hours, at least in in vitro model systems. By judicious administration of activation signals refractory cells can be reactivated and one might expect that this process could occur in the intact animal, although this juncture might represent a beginning breakdown of host defense mechanisms.

Macrophages would be expected to maintain some level of activation for longer periods of time and might continue to secrete monokines, phagocytize cellular debris, and release lysosomal enzymes,

proteases, or other cellular enzymes, such as arginase. Some of these enzymes may contribute to subsequent tumor killing.

One may surmise that events in this model are occurring naturally in the tumor bearing animal and that agents such as BCG and endotoxin merely stimulate them so that tumor killing becomes dramatic even in the presence of tumor products which may tend to suppress host defenses. In this context tumor necrotizing factor is only one component of a complex sequence of events. When present it seems to play a paramount role. Methods to stimulate TNF production in a tumor bearing host without the concomitant use of immunopotentiators and endotoxin are important if it is to be used clinically.

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## BIOGRAPHICAL SKETCH

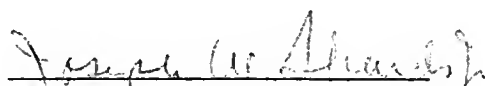
Michael stumbled into this best of all possible worlds in anno Domini MCMLIII, New Orleans, Louisiana. Proud son of his even prouder parents, he quickly learned how to dismantle the lawnmower and TV. This compelled his parents to send him to school where he met other radical young boys and spent many carefree childhood weekends in the country assembling pyrotechnics and incendiaries. After high school in Camden, New Jersey, at the impressionable age of eighteen, his parents coughed up thousands of dollars to send him to the prestigious Mid-Atlantic university, Johns Hopkins, to stay out of trouble and make something of himself. This he failed to accomplish. He did, however, learn to play lacrosse and finally received a Bachelor of Arts in biophysics. Upon matriculation in the fall of 1975, he assumed a position in the Department of Immunology and Medical Microbiology media kitchen. There he successfully completed the degree of Doctor of Philosophy in March, 1980. Michael is presently unemployed and looking for a job. He was last sighted heading for Colorado.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



George E. Gifford, Ph.D., Chairman  
Professor of Immunology and  
Medical Microbiology

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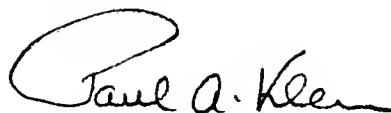
Joseph W. Shands Jr., M.D.  
Professor of Immunology and  
Medical Microbiology

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R. Michael Roberts, Ph.D.  
Professor of Biochemistry

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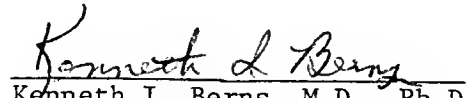
Paul A. Klein, Ph.D.  
Associate Professor of Pathology

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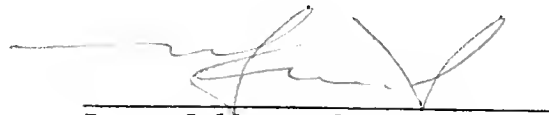
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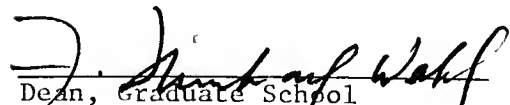
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